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(54) Title: USE OF SMAD 7 ANTISENSE OLIGONUCLEOTIDES (ODN) FOR THE TREATMENT OF DISEASES MEDIATED BY THE NUCLEAR TRANSCRIPTION FACTOR NF-KB

(57) Abstract: The present invention refers to the use of antisense oligonucleotidic sequences (ODN) for Smad7 opportunely modified for the treatment of Inflammatory and tumour pathologies mediated by the nuclear transcription factor NF-kB, such as gastric carcinoma correlated to *Helicobacter pylori* Infection.

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USE OF SMAD7 ANTISENSE OLIGONUCLEOTIDES (ODN) FOR THE TREATMENT OF DISEASES MEDIATED BY THE NUCLEAR TRANSCRIPTION FACTOR NF-KB.

The present invention refers to the use of Smad7antisense oligonucleotides (ODN) for the treatment of diseases mediated by the nuclear transcription factor NF-kB.

In particular the invention refers to the use of Smad7 antisense oligonucleotides (ODN) for the treatment of chronic inflammatory diseases and tumours that result to be associated with an abnormal and increased activity of the nuclear transcription factor NF-kB. In this context, an example is represented by *Helicobacter Pylori (Hp)* -associated diseases, such as gastric carcinoma.

The NF-kB family includes proteins involved in the regulation of multiple intracellular processes that are relevant either in the mechanisms of inflammation or apoptosis and cell death. This mostly relies on the ability of NF-kB to regulate the transcription of various molecules (e.g. cytokines, adhesion proteins, surface receptors and caspases).

The NF-kB proteins are present in the cytoplasm of most cells in homodimeric or heterodimeric complexes. Each NFkB component contains an amino terminal region necessary for binding to the DNA, a dimerization domain, and a sequence necessary for the protein translocation into the nucleus (NLS).

NF-kB is located in the cytoplasm of non-stimulated cells in an inactive form due to the interaction with IkB, a class of inhibitory proteins. Such proteins interacts with NF-kB components and mask the NLS sequence thus preventing the translocation into the nucleus.

In response to activating stimuli, such as those generated during bacterial infections, inflammatory or neoplastic processes, IkB is phosphorylated by intracellular kinases denominated IkK, and eventually degraded. This allows NF-kB to migrate into the nucleus, where it interacts with DNA targets, and regulates the transcription of multiple genes, that encode for cytokines and proInflammatory enzymes (e.g TNF-α, IL-1, I-CAM-1, IL-8, COX-2, iNOS) and/or other molecules involved in the inflammatory response underlying the aetiology of several pathologies. Among the genes induced by NF-kB there is IkBα. In other words, the

activation of NF-kB also results in the synthesis of IkBα, which in turn suppresses NF-kB signalling.

Despite the existence of this negative feed-back loop, that is able to regulate the level of NF-kB activation, an excessive activity of the transcription factor has been documented in a variety of human inflammatory diseases, such as *Helicobacter pylori*-associated chronic gastritis, bronchial asthma, and auto-immune diseases, including rheumatoid arthritis, psoriasis and multiple sclerosis, neuro-degenerative pathologies such as the Alzheimer's and Parkinson's disease, chronic intestinal Inflammatory diseases (IBD) (Li Q. et al.., 2002; Zingarelli et al.., 2003), and many neoplastic diseases such as gastric and colonic carcinoma and lymphoma, some of the principal tumours of the digestive tube in the Western World (Sasaki et al. , 2001; Yu HG et al., 2003). For each of these pathologies, the mechanisms underlying the persistent NF-kB activation remain unknown.

It is however conceivable that the activation of the transcription factor could rely either on the sustained stimulation by inflammatory cytokines (e.g. IL-1, TNF-α), viral and bacterial products (e.g. lipopolysaccharides), or on reduced activity of counter-regulatory molecules, such as TGF-β1.

With regard to the action of stimulatory molecules, it has been recently shown that *Hp* activates NF-kB in gastric epithelial tumoural cellular lines, by involving different kinases, such as IKKs (Maeda et al., 2001) and a kinase inducing NF-kB (NIK) (Maeda et al., 2000). Conversely, TGF-β1 could have an essential and negative role in the control of NF-kB activity. Indeed, animals with either targeted deletion of TGF-β1 gene or resistant to the action of this protein exhibit an increased expression of inflammatory molecules that could depend on NF-kB activity. Such data are consistent with the demonstration that, in experimental models, a defective activity of TGF-β1 can associate with the development of inflammatory or neoplastic diseases, which show immuno-morphological similarities with human diseases characterized by increased NF-kB activity (Strober et al., 2002, Han et al., 1998). In the specific case of gastritis, TGF-β1 knock-out mice develop lesions that are similar to those caused by *Hp* Infection, such as hyperplasia and gastric dysplasia.

There is also evidence that, in some of these models, restoration of TGF-β1 activity results in a decreased NF-kB activity and resolution of the lesions.

Based upon these data, it is thus conceivable that a defective TGF-β1 activity can contribute to a sustained activation of NF-kB.

The author of the present invention has shown, in a previous study, that a defective activity of TGF-β1 in Crohn's disease is also associated with high expression of Smad7 (Monteleone et al., 2001). Smad7 is a protein that, interacting with the type I TGF-β1 receptor, prevents binding and phosphorylation of Smad2/3 (Hayashi et al., 1997), thereby inhibiting Smad2/3 translocation into the nulceus.

It is thus conceivable that high Smad7 protein expression can culminate in a sustained activity of NF-kB due to the inhibition of the TGF-β1-mediated suppression.

About half of the world's population is infected with *Helicobacter pylori (Hp)*, a Gram-negative bacterium that colonises the mucosal layer of the gastric epithelium (Ernst et al., 2000). There is enormous heterogeneity in the consequences of infection. The Infection inevitably causes a chronic gastric inflammation. The majority of infected individuals are asymptomatic. However a minority of patients develop clinically relevant gastritis, and some of these go on to develop gastric ulcers, gastric carcinoma or a low-grade B cell lymphoma. In HP-infected patients and peptic ulcer, eradication of bacterium represents an essential therapeutic objective for healing of the lesions. In fact, the persistent mucosal Hp colonisation is inevitably accompanied by a recurrence of ulcer. However such a therapeutic goal is not easily achievable, because about one third of HP-infected patients are resistant to antibiotic treatment.

The reasons for this heterogeneous response are not known, but it appears to be controlled by both bacterial and host factors (Ernst et at 2000, Israel et al., 2001). For example, infection of gastric epithelial cells with Hp activates NF-KB and induces the secretion of chemokines such as interleukin (IL)-8, which attract neutrophils into the tissue (Chu et al., 2003, Bhattacharyya et al. 2002). The host also mounts an adaptive immune response. There is local production of anti-Hp IgA and IgG and importantly, there is a local Th1 cell response with increased synthesis of interferon (IFN)-γ, TNF-α and IL-12 (Ernst et al., 2000, Israel et al., 2001, D'Elios et al., 1997). While it is difficult to assess whether the Th1 response contributes to tissue injury in patients, animal models of Hp

infection clearly show that Th1 cells are essential in the development and maintenance of lesions associated with HP-infection (Smythies et al., 2000, Eaton et al., 2001). The mechanism by which Th1 cell inflammatory response is generated and maintained in the Hp-infected gastric mucosa remains however unclear. It is however conceivable that changes in the mechanisms involved in the control of the gastro-intestinal homeostasis can have a decisive role in the pathogenesis of Hp infection -associated lesions. A possibility is that a defective activity of TGF-β1, a molecule that normally suppresses both Th1-cell-mediated immune responses and NF-kB activity (Letterio et al., 1998, Wahl et al., 1994), plays an essential role in the cascade of events that contribute to the Hp infection -associated gastric pathology. It is also possible that defective TGF-β1-mediated signal transduction can influence the onset of additional pathologies correlated to excessive activation of NF-kIB (Hahm et al., 2002).

The therapeutic intervention in diseases characterised by increased activity of NF-kB is mostly symptomatic, and in most cases not curative. It is often surgical and invasive, and despite that not decisive for most of cancer patients. The use of molecules that selectively and specifically regulate one or more phases essential for the development and perpetuation of such pathologies could thus represent a promising and valid alternative to the currently available pharmacological therapies.

The author of the present invention has now shown that opportunely modified Smad7 antisense oligonucleotides can be advantageously used for controlling NF-kB activity in diseases associated with a dys-regulation of this intracellular pathway. Such an approach would moreover offer the advantage to facilitate the TGF-β1-mediated suppression of additional intracellular mechanisms, that contribute to the maintenance of inflammatory and neoplastic processes, such as activity of STAT molecules, MAP kinase, or regulation of cell death.

The antisense oligonucleotides (ODN) are short oligonucleotidic sequences complementary to the messenger RNA (mRNA) which encodes for the protein target (it is Smad7 in the present case). Such sequences, coupling with the m-RNA, form a hybrid double strand chain, which causes the activation of ubiquitous catalytic enzymes, such as RNases H, that eventually degrade the hybrid DNA/RNA chains thereby preventing the protein translation.

The author of the present invention now shows that wherever Smad7 is over-expressed there may be a sustained activation of NF-kB,

due to the defective suppression mediated by TGF-β1. It is also noteworthy that in some cell types, NF-kB antagonises the biological action of TGF-β1 by enhancing Smad7 itself. This would trigger a positive feed-back loop which eventually amplified NF-kB signalling.

The experiments performed by the author of the present invention show a marked reduction of active Smad3 levels in gastric biopsies taken from patients with *Hp* Infection, despite the normal production of TGF-β1 and increased expression of TGF-β1 receptors. The reduction of phosphorylated Smad3 is associated with a marked expression of Smad7 both in epithelial and in the propria lamina mononuclear cells (LPMC).

The *in vitro* treatment of the gastric biopsies with the Smad7 antisense oligonucleotides reduces the levels of Smad7 protein, enhances Smad3 activation and leads to a reduction in the expression of Inflammatory Th1 type molecules such as T-bet and IFN-γ. It is also shown that IFN-γ induces Smad7 in gastric biopsies of normal subjects, whereas neutralisation of IFN-γ activity reduces Smad7 expression in the gastric mucosa of Hp-infected patients.

Together these data suggest that, during Hp infection, gastric Th1 type cytokines stimulate the induction of Smad7, that in turn blocks the TGF-β1-mediated immune-suppression, thereby contributing to expand the ongoing mucosal inflammation and perhaps the tissue damage.

The authors has also performed experiments using biological samples from additional pathologies associated with an altered activation of NF-kB, such as chronic intestinal Inflammatory diseases (IBD).

In this contest, it has been shown the ability of TGF-β1 to negatively regulate NF-kB in normal human intestinal LPMC and the correlation between the reduced activity of TGF-β1, due to increased expression of Smad7, and the chronic activation of NF-kB in LPMCs from patients with IBD. Particularly, it has been shown that, in LPMCs isolated from the intestine of normal subjects, TGF-β1 causes a marked suppression of the translocation and accumulation of NFkB/p65 in the nucleus, the binding of NF-kB to DNA target and activation of NF-kB-dependent genes induced by stimulation with TNF-α.

In marked contrast, in LPMCs isolated from patients with IBD, TGF-β1 does not reduce the activation of NF-kB induced by TNF-α. However, in the same cells, the inhibition of Smad7 expression, by the use

of Smad7 anti-sense oligonucleotides associates with a reduction in the accumulation of NFkBp65 in the nucleus and increased expression of IkBα. These studies, carried out in experimental models of Inflammatory pathologies mediated by the activation of NF-kB, identify Smad7 as a possible biological target to control NF-kB activation.

Therefore it is an object of the present invention the use of antisense phosphorothioate oligonucleotides for Smad7 up to 21 nucleotides in length comprising a portion of at least 10 nucleotides of the following sequence:

5'-GTXYCCCCTTCTCCCXYCAGC-3 ' (SEQ ID No 1)

wherein X is a nucleotide comprising a nitrogen base selected from the group consisting of cytosine, 5-methylcytosine and 2'-O-methylcytosine and wherein Y is a nucleotide comprising a nitrogen base selected from the group consisting of guanine, 5-methylguanine and 2'-O-methylguanine, with the provision that at least one of nucleotides X or Y comprises a methylated nitrogen base or its complementary sequence, for the preparation of a drug useful for the treatment of diseases mediated by an altered activation of the nuclear transcription factor NF-kB.

It is also considered the use of oligonucleotidic sequences of the several stereoisomers of antisense oligonucleotides according to the present invention, such as diastereoisomers and enantiomers, with respect to the phosphorus atoms of the internucleosidic bonds present in the sequence.

The antisense oligonucleotides used according to the present invention can have at least one nucleotide of the sequence methylphosphonated, for instance, at only one end 5' or 3' or at both ends 5' and 3' or along the oligonucleotidic sequence.

In a preferred form the methylphosphonated nucleotide can be X or Y, so that the methylphosphonated internucleosidic bond is the bond between the indicated nucleotides.

The antisense oligonucleotides used according to the present invention can further have at least one nucleotide of the sequence that is a 2'-O-methylribonucleotide 5'-monophosphate, for instance, only at one end 5' or 3' or at both the ends 5 ' and 3 ' or along the oligonucleotidic sequence.

It is a further object of the present invention the use of the antisense oligonucleotides as described above wherein 2'-deoxiribonucleotides are replaced with ribonucleotides and 2'-

deoxithymidine is replaced with uridine so that the deoxiribonucleotidic sequences are replaced by corresponding ribonucleotidic sequences.

A preferred form of the present invention is represented by the use of an antisense oligonucleotide having the sequence:

5'-GTXGCCCCTTCTCCCXGCAGC-3 ' (SEQ ID No 3)

wherein X is 5-methyl 2'-deoxicytidine 5'-monophosphate.

An additional preferred form is the use of an antisense oligonucleotide having the sequence:

5'-ZTXGCCCCTTCTCCCXGCAZ-3 ' (SEQ ID No 2)

wherein X is 5 methyl 2'-deoxicytidine 5'-monophosphate and Z is 2'-deoxiguanosine methylphosphone.

The invention is referred to the use of the aforesaid antisense oligonucleotidic sequences for the preparation of a drug for the treatment of Inflammatory and tumoral pathologies mediated by altered activation of the nuclear transcription factor NF-kB.

Particularly, such Inflammatory pathologies are selected from the group consisting of *Helicobacter pylori* Infection-associated diseases, bronchial asthma, rheumatoid arthritis, psoriasis, multiple sclerosis, Alzheimer's disease, Parkinson's disease, intestinal chronic Inflammatory diseases (IBD) and neoplastic pathologies.

With regard to the neoplastic pathologies in which the treatment with the indicated Smad7 antisense oligonucleotides is aimed at regulating NF-kB activation and expression of NF-kB-dependent genes, there is a preferential choice for carcinoma and lymphoma, particularly those associated with *Helicobacter pylori* Infection.

The present invention will be now described by way of illustration, but not limitation, according to the preferred forms of realization, and with particular regard to figures of the enclosed drawings, wherein:

figure 1 shows the ELISA assay of active TGF-β1 at level of the gastric mucosa colonised from *Helicobacter pylori* performed on total protein extracted from gastric biopsies of 8 Hp-positive and 8 Hp-negative patients. Each point represents the value of active TGF-β1 in mucosal samples collected from a single individual and the horizontal bars indicate the median value;

figure 2 shows immunohistochemical staining for the type I and type II TGF-β1 receptors in paraffin sections of gastric tissue from Hp-positive and Hp-negative patients. In both right panels, it is shown the

immunohistochemical staining with a control rabbit isotype IgG corresponding to the gastric sections of Hp-positive patients shown in the left panels (X 40 magnification). The example is representative of 4 separate experiments, analysing in total gastric biopsies from 8 Hp infected and 7 Hp-negative patients;

figure 3 shows reduced phosphorylation of Smad3 in Hp-colonised gastric mucosa.

Panel A. Representative expression of both phosphorylated (p-Smad3) and total Smad3 protein in biopsies from 3 Hp-positive and 3 Hp-negative patients. The example is representative of 4 separate experiments analysing biopsies from 13 Hp-positive and 13 Hp-negative patients.

Panel B. Quantitative analysis of the active/inactive Smad3 ratio in gastric biopsies from the 13 Hp-positive and 13 Hp-negative patients, measured through densitometric analysis of the Western Blots. The values are expressed in arbitrary units (u.a.) and each point represents the value (u.a.) of the active/inactive Smad3 ratio in the biopsy taken from on a single individual, while the horizontal lines indicate the mean value. Some samples have very similar levels of u.a. so that only 8 points for every group are evident in figure.

Panel C. Representative expression of p-Smad3 and total Smad3 proteins in lamina propria mononuclear cells (LPMC) isolated from the gastric mucosa of 3 Hp-positive and 3 Hp-negative patients. The example is representative of two separate experiments analysing LPMC samples from 5 Hp-positive and 5 Hp-negative patients;

figure 4 shows the increase of Smad7 expression in gastric biopsies of patients infected with Hp Infection.

Panel A. Representative expression of Smad7 and β-actin proteins in gastric biopsies taken from 3 Hp-positive and 3 Hp-negative patients. The example is representative of four separate experiments analysing in total biopsies from 13 Hp-positive and 13 Hp-negative patients.

Panel B. Quantitative expression of Smad7 in samples as measured by densitometric analysis of the Western Blots. The values are express in arbitrary units (u.a.) and each point represents the value (u.a.) of the content of Smad7 in mucosal samples taken from each single individual, while the horizontal lines indicate the mean value.

Panel C. Representative expression of Smad7 and β-actin proteins in lamina propria mononuclear cells (LPMC) isolated from the gastric mucosa of 3 Hp-positive and 3 Hp-negative patients.

The example is representative of two separate experiments analysing LPMC samples from 5 Hp-positive and 5 Hp-negative patients.

Panel D. Expression of Smad7 and β-actin in patients with Hp Infection before (B) and after (A) a successful eradication therapy. The gastric mucosal samples were taken from the antrum of three patients with Hp Infection. The example is representative of two separate experiments analysing samples from 5 patients;

figure 5 shows the induction of Smad7 by IFN-γ in gastric mucosa.

Panel A. Representative expression of Smad7 (upper blot) and β-actin (lower blot) proteins in gastric mucosal samples collected from Hp-negative patients, and cultured for two hours in medium alone (UNS) or in the presence of filtrates of cultural broth of *Hp* or *Brucella* (control), or 100 ng/ml recombinant IFN-γ (rh-IFN-γ). The example is representative of three separate experiments analysing gastric mucosal samples from 4 Hp-negative patients.

Panel B. RT-PCR gel showing IL-8 transcripts in AGS cells cultured for one hour in medium alone (UNS), or in the presence of filtrates of cultural broth of *Hp* or *Brucella* (control), or 100 ng/ml recombinant IFN-γ (rh-IFN-γ).

Panel C. Neutralisation of endogenous IFN-γ inhibits Smad7 in *Hp*–infected gastric mucosal samples. Western Blot showing Smad7 protein in gastric mucosal samples collected from Hp-positive patients, and subsequently cultured for 24 hours in medium alone (UNS) or in presence of an IFN-γ neutralising or control antibody, respectively. The example is representative of three separate experiments analysing mucosal samples from 4 Hp-positive patients.

figure 6 shows the interaction between IFN-γ and STAT1 protein.

Panel A. IFN-γ enhances STAT1 activation in normal gastric mucosa. Normal gastric biopsies were pre-incubated with Tyrphostin B42 (TB42), an inhibitor of JAK2-STAT1, or DMSO for 30 minutes before the stimulation with IFN-γ (100 ng/ml) for 1 hour. UNS = biopsies cultured in medium alone. Total proteins were analysed for the content of p-Tyr-STAT1 (upper blot) and of Total STAT1 (lower blot). The example is

representative of three separate experiments analysing gastric mucosal samples from 4 normal control patients.

Panel B. Inhibition of STAT1 activation prevents the IFNγ-mediated induction of Smad7. Normal gastric biopsies were pre-incubated with Tyrphostin B42 (TB42) or DMSO for 30 minutes before the stimulation with IFN-γ (100 ng/ml) for 2 hours. Smad7 and β-actin proteins were then analysed by Western blotting. One of three experiments, analysing in total mucosal samples from 4 Hp-negative patients, is shown;

figure 7 shows Smad7 inhibition with a specific antisense oligonucleotide (AS) in Hp-infected gastric mucosal samples.

Panel A shows the increase in p-Smad3 and decrease in the expression of both IFN-γ and T-bet.

Panel B. Gastric biopsies collected from a Hp-positive patient cultured in medium alone (M), in presence of a control sense oligonucleotide (S), or a specific Smad7 antisense oligonucleotide (AS), for 24 hours. The example is representative of 4 separate experiments, in which gastric mucosal biopsies from 4 Hp-positive patients were used. Identical results were obtained in each experiment.

Panel C. Inhibition of IFN-γ secretion in supernatants of organ cultures of biopsies from Hp-infected patients by Smad7 antisense oligonucleotide (AS);

figure 8, panel A shows the TGF-β1-mediated inhibition of nuclear translocation of NF-kB/p65 induced by TNF-α in normal LPMC. Western blot shows NF-kB/p65 in nuclear extracts from normal LPMC (inset). Quantitative analysis of the NFkBp65/histone-1 ratio. Each point represents a single subject (n=5/group); while figure 8, panel B, shows Western blot of NF-kB/p65 in cytosolic extracts from the same normal LPMC;

figure 9 shows the inhibition of TNF-α-induced NF-kB binding activity by TGF-β1 in normal LPMC. Representative EMSA showing the bonding activity of NF-kB to DNA. One of the five representative experiments is shown;

figure 10 shows the TGF-β1-mediated inhibition of IL-8 RNA production induced by TNF-α in normal LPMC.

Panel A. Agarose gel showing IL-8 and β-actin transcripts. One of four representative experiments is shown.

Panel B. Quantitative analysis of IL-8/β-actin ratio (N=4 patients, mean ±1 SEM);

figure 11, panel A, shows as the pre-treatment with TGF-β1 inhibits the degradation of IkBα protein induced by TNFα in normal LPMC; figure 11, panel B, shows the quantitative analysis of the IL-8/α-actin ratio (N=5 patients, mean ±1 SEM); figure 11, panel C, shows the increase of IkBα protein in normal LPMC induced by TGF-β1. Western blot shows IkBα in cytosolic extracts from normal LPMC cultured in medium for 7 hours and with TGF-β1 for the indicated time points. One of five experiments is shown. Quantitative analysis of IkBα/β-actin (N=5 patients); figure 11, panel D, shows the TGF-β1-mediated induction of IkBα transcription in normal LPMC. Agarose gel shows both IkBα and β-actin transcripts in normal LPMC;

figure 12 shows the TGF-β1 effect on the activity of the IkBα promoter in primary fibroblasts isolated from human foetal gut. Luciferase activity was analysed and normalised to the activity of pRLTK. It was then expressed as entity of the induction in comparison to the untreated control sample. Data indicate mean ± 1 S.E.M of three independent transfections;

figure 13, panel A, shows the Western blot of NF-kB/p65 in nuclear extracts from LPMC isolated from the colon of 5 IBD patients and and 5 normals. One of two experiments analysing in total LPMC from 5 patients with Crohn's disease, 3 with ulcerative colitis and 8 normals is shown. Inset shows the quantitative analysis of the p65/histone1 ratio (N=8 patients/group). Figure 13, panel B, shows the Western blot of IkB-α in cytosolic extracts from LPMC isolated from the colon of 5 IBD patients (3 CD and 2 UC) and 5 normals. One of two representative experiments analysing cells from 5 CD, 3 UC and 8 samples is shown. The blot shows IkBα in the same LPMC samples analysed for nuclear NF-kB (panel A). Inset shows the quantitative analyses of the IkBα/β-actin ratio (N=8 patients/group);

figure 14 shows the lack of TGF-β1-mediated inhibition of NF-kB activation in IBD LPMC.

Panel A. Western blots showing NF-kB/p65 in nuclear extracts from IBD LPMC. Inset shows the quantitative analysis of the p65/histone1 ratio (N=8 patients/group).

Panel B shows the two representative EMSA blots showing the bonding activity of NF-kB to DNA in IBD LPMC;

figure 15, panel A, shows the effect of treatment of IBD LPMC with an antisense oligonucleotide for Smad7 or control sense oligonucleotide. The antisense but not the control DNA inhibits Smad7

expression. The arrows indicates Smad7 protein as revealed by a specific polyclonal antibody. The example is representative of three separate experiments analysing in total LPMC from four IBD patients (3 CD and 1 UC). The quantitative data of this experiment are shown in the inset. Each point indicate the quantity of Smad7 in LPMC isolated from a single subject (n=4/group).

figure 15, panel B, shows that the inhibition of Smad7 increases IkBα expression in IBD LPMC. Inset: quantitative analysis of IkBα/β-actin ratio in LPMC from four IBD patients (3 CD and 1 UC). Each point represents a single subject (n = 4/group). Figure 15, panel C, shows that the inhibition of Smad7 is accompanied by accumulation of NF-kB/p65 at the nuclear level in IBD LPMC. Inset: quantitative analysis of p65/histone-1 ratio in LPMC from 3 CD and 1 UC. Each point represents a single subject (n=4/group);

figure 16 shows the inhibition of TNF-a-induced NF-kB activation by Smad7 antisense oligonucleotides and its dependence on TGF-β1 activity in IBD LPMC. The basal binding activity of NF-kB is reduced in comparison with cells pretreated with medium or sense oligonucleotide for Smad7;

figure 17 shows PCR products for Smad7 in gastric (AGS and MKN 28) and colonic (HT-29, HT115, T84, Caco2) epithelial cancer cell lines.

EXAMPLE 1: *Induction and regulation of Smad7 in the gastric mucosa of Hp-infected patients.*

MATERIALS AND METHODS

Patients and samples.

44 patients (20 men and 24 women, median age 42, range from 21 to 74 years) who underwent esophagogastroduodenoscopy for dyspeptic symptoms were studied.

Among these individuals, 22 patients were Hp positive as confirmed by urease quick test and histology. Five Hp-infected patients underwent a further endoscopy 2 months after the suspension of eradicating treatment consisting in the administration, for a week, of omeprazole(20 mg twice daily), amoxicilin (1000 mg twice daily) and claritromycin (500 mg twice daily). In all these five patients, eradication was confirmed by C-13 labelled urea test and histology.

22 of the 44 patients were Hp-negative. Mucosal samples, which were macroscopically and histologically normal, were taken from all

22 patients. During endoscopy, at least six gastric biopsy specimens were taken: one from the antrum for urease quick test, four from the antrum and corpus for the histological and immunohistochemical test and the residual samples from the antrum for the measurement of TGF-β1 and of Smad3 and Smad7 expression.

Additional biopsy specimens were taken from the gastric antrum of 8 Hp-positive and 4 Hp-negative patients and used for the preparation of the organ cultures. None of the enrolled patients had received antibiotics within the 2 months preceding the study. The informed consent was obtained from all patients and the protocol was approved by the local ethical committee.

Organ cultures and gastric LPMCs isolation

Mucosal biopsies were cultured as described in Fais et al., 1992. Briefly, the biopsies collected from the gastric antrum of 4 Hp-negative patients were placed on iron grids with the mucosa layer oriented upwards in a Petri dish and immersed in a serum free medium containing RPMI 1640 (Sigma, Milan) supplemented with 10% HL-1 (BioWhittaker, Verviers, Belgium), penicillin (100U/ml) and streptomycin (100 μg/ml) (LifeTechnologies-GibcoBRL, Milan). Cultures were performed with or without the addition of a broth culture filtrate derived from Hp 60190 strain (wild type) or a *Brucella* control strain (final dilution 1:3) (Ricci et al., 1996) or the addition of recombinant IFN-γ (rh-IFN-γ, final concentration 100 ng/ml) (Peprotech EC LTD, London) for 1-4 hours. Further, the cultures were stimulated with rh-IFN-γ in the presence or absence of a Jak2/STAT1 inhibitor such as Tyrphostin B42 (TB42) (100 μM, Inalco S.p.A., Milan) or DMSO (Sigma) for a period varying from 30 up to 120 minutes. TB42 and DMSO were preincubated for 30 minutes before the addition of rh-IFN-γ. Finally, the gastric biopsies collected from 4 Hp-positive patients were cultured in the presence or absence of a control or IFN-γ neutralising antibodies (5 μg/ml, R&D Systems, Abingdon, UK) for 24 hours and examined for the content of Smad7.

Additionally the biopsies collected from the antrum of 4 Hp-positive patients were cultured in the presence or absence of Smad7 sense and antisense oligonucleotides (10 μg/ml). Both Smad7 sense and antisense oligonucleotide were combined with 2000 Reagent lipofectamine (Invitrogen Italy, St. Giuliano Milanese) 20 minutes before using, according to the instructions of the manufacturer. The characteristics of both oligonucleotides have been described in previous

studies (Monteleone et al., 2001). Briefly, the phosphorothioate single strand oligonucleotides match the region 107-128 (5'-GCTGCGGGGAGMGGGGCGAC-3') of the human Smad7 complementary DNA sequence and were synthesized in the sense and antisense orientation. The Petri dishes with the organ cultures were placed in a hermetic container with 95% O_2 / 5%CO_2 at 37°C at 1 bar. After 24 hours, the total proteins were extracted from tissue and analysed for the expression of cytokines by Western Blotting. The supernatants of the organ cultures were collected and used to measure IFN-γ by ELISA (Peprotech).

Gastric LPMCs were isolated by standard methods including the sequential use of DTT, EDTA and collagenase as previously described in detail in previous studies (Monteleone et al., 1997). The percentage of epithelial cells contaminating LPMC preparations was <5% in any case.

The isolated cells were counted and checked for viability, using 0.1% trypan blue solution (this was between 89 and 93%)..

Cell line coltures, RNA extraction, preparation of the complementary DNA (cDNA) and RT-RCR.

The gastric epithelial cell line, AGS, (Barranco et al. ,1998), was grown in complete medium DMEM F-12 (BioWhittaker) supplemented with 10% foetal bovine serum (FBS) at 37°C in an humidified atmosphere with 5% CO_2 in air. The sub-confluent layers of AGS cells in 6 well-plates were starved overnight and subsequently incubated in the presence or absence of broth cultural filtrate derived from a Hp 60190 strain (wild-type) and a control (final dilution 1:3) or rh-IFN-γ (final concentration 100 ng/ml) for 1 to 4 hours. Finally, the cells were collected and used for RNA extraction. Total RNA, cDNA preparation and RT-PCR were carried out as previously described (Monteleone et al., 1997). PCR primers were as follow:

IL-8, FWD: 5'-TGCAGCTCTGTGTGAAGG-3 ' (SEQ ID No 4);
REV: 5 ' - ATTGCATCTGGCMCCCTAC-3 ' (SEQ ID No 5);
β-actin, FWD: 5'-GGCACCACACCTTCTACA-3 ' (SEQ ID No 8);
REV: 5'-CAGGTCTTTGCGGATGTC-3 ' (SEQ ID No 9).

TGF-β1 ELISA

Frozen biopsies were homogenised in liquid nitrogen and the total protein extract were collected in buffer containing 10 mM Hepes (pH 7,9), 10 mM KCl, 0,1 mM EDTA and 0,2 mM EGTA supplemented with 0,1 mM Dithiothreitol (DTT), aprotinine (10 μg/ml), leupeptine (10 μg/ml) and 1 mM phenylmethansulphonyl fluoride (all the reagents from Sigma). One

hundred μg of total proteins per sample were analysed using a commercial ELISA kit (R&D Systems), according to the instructions of the manufacturer. Values of active TGFβ1 were expressed as pg/100 μg of total proteins.

Immunohistochemistry

Tissue sections from 8 Hp-positive and 7 Hp-negative patients were cut, deparaffinised and dehydrated after treatment with xylene and ethanol and then the slides were incubated in microwave oven for 20 minutes in 0,01 Mm citrate buffer, pH 6 (Sigma). The incubation with anti-TGF-β1 RI (Novocastra Laboratories Ltd, Newcastle, UK) or RII antibodies (C-16 Saint Cruz Biotechnology, Saint Cruz, CA, USA), used at a dilution 1:20, at 4°C was performed overnight. Staining specificity was confirmed by using blocking peptides. After rinse in TBS (Sigma), the slides were incubated with a secondary antibody conjugated to HRP peroxidase (dilution 1:50, Dako SpA, Milan) for 30 minutes at room temperature.

Immunoreactive cells were visualised by addition of diaminobenzidine (Sigma) as substrate and counterstained with haematoxylin. The control sections were prepared under the same immunohistochemical conditions, as described above, replacing the primary antibody with a control antibody (Dako). Following dehydration by treatment with xilene and alcohol the slides were mounted and analyzed by optical microscopy.

Western blotting

Western blotting analysis was performed on gastric biopsies collected from 14 Hp-positive and 14 Hp-negative patients. Total proteins were prepared as above described.

For the detection of phosphorylated Smad3 (p-Smad3), 200 μg/sample of total proteins were separated on a 10% SDS-PAGE gel. Phosphorylated Smad3 was detected using a rabbit anti-human pSmad2/3 (final dilution 1:500) (Saint-Cruz biotechnology) and followed by a HRP-peroxidase conjugated goat anti rabbit IgG (final dilution 1:10000).

The reaction was detected with a chemiluminescence kit (West Dura, Pierce, Rockford, IL, USA). Following the analysis of phosphorylated Smad3, blots were stripped and incubated with a rabbit anti human Smad3 antibodies (2 μg/ml, Upstate, Lake Placid, NY, USA) and subsequently with goat anti-rabbit antibody conjugated to HRP-peroxidase (dilution 1:10000).

Smad7 was analyzed using a specific rabbit anti-human Smad7 antibody (H-79; final dilution 1:500, SantaCruz Biotechnology). Goat anti rabbit antibodies conjugated to HRP-peroxidase (dilution 1:50000) were used to detect the antigen-antibody binding and the immunoreactivity was visualized as above described.

IFN-γ and T-bet were analyzed using specific goat anti-human IFN-γ and anti-T-bet antibodies (dilution final 1:500, Saint Cruz Biotechnology). Rabbit anti-goat antibodies conjugated to HRP peroxidase (dilution 1:20000) were used to detect primary antibodies binding and the immunoreactivity was visualised as above described. In order to ascertain equivalent loading and proteins transfer, the stain was performed with ponceau S (Sigma).

Furthermore the blots were stripped and analyzed for the content of β-actin, as internal control, using specific mouse anti human β-actine antibodies (dilution 1:5000, Sigma), followed by goat anti-mouse antibodies conjugated to HRP peroxidase (dilution 1:30000).

To investigate STAT1 expression, total proteins (250 μg) were separated on 8% SDS-PAGE gel and firstly analyzed for the content of phosphorylated STAT1 (p-STAT1) using a mouse antibody that specifically recognises the phosphorylation of human STAT1 on tyrosine 701 residue (final dilution 1:1000; Saint Cruz Biotechnology). Rabbit anti-mouse antibodies conjugated to HRP peroxidase (dilution 1:20000, Dako) were used as secondary antibodies and the reaction detected as above described.

After detection of p-STAT1, blots were stripped and incubated with rabbit anti human polyclonal antibodies that recognise the total STAT1 forms (dilution final 1:2000, Saint Cruz Biotechnology) followed by a goat anti-mouse antibodies conjugated to HRP-peroxidase (dilution 1:20000, Dako).

The intensity of bands was analyzed by densitometry using a computer assisted system (Total lab, AB.EL Sience-Ware Srl, Rome).

Statistic analysis

Differences between groups were compared using either the Mann-Whitney U test for not normally distributed data, or the Student test if the observations were consistent with a sample deriving from a normally distributed population.

RESULTS

TGF-β1 negatively regulates mucosal immune response and a defective production of TGF-β1 has been associated with the development of gastritis in experimental models (Letterio et al., 1998, Yang et al., 1999). The authors have analysed if Hp infection-related gastritis is associated with changes in the production of active TGF-β1. Since severed gastric mucosal cell types are able to synthesize TGF-β1, the cytokine was measured in total protein extracts from whole gastric mucosa rather than in single purified cell types.

Using a commercially available ELISA kit, suitable to detect the content of the active TGF-β1, it has been shown that the cytokine was expressed in all the samples independently from the presence or not of Hp Infection. Although some gastric samples from Hp-positive individuals exhibited higher levels of TGF-β1 than those observed in samples from Hp-negative individuals, the median value did not differ significantly between the two groups as shown in figure 1 (62 pg/100μg total proteins, range 34-124, in infected patients versus 44pg/100 μg total proteins, range 27-82, in patients not afflicted by Hp Infection, respectively).

On the contrary, the immunohistochemistry revealed that the expression of both TGF-β1 RI and RII receptors is increased in gastric mucosa during Hp Infection (figure 2).

Such an increase was particularly evident at level of LPMCs, even if an increased staining was also observed in Hp-colonized epithelial cells. The specificity of data was confirmed using blocking peptides and a non relevant isotype antibody (figure 2, right panels).

The TGF-β1-mediated signal transduction mechanism uses Smad proteins (Heldin et al., 1997, Derynck et al., 1998). Recent studies have show that the anti-inflammatory activity of TGF-β1 the mucosal level is closely associated to its ability to induce the phosphorylation and activation of Smad3 (Yang et al., 1999, Monteleone et al., 2001).

Based on these considerations it has been further assessed whether the high expression of TGF-β1 receptors was associated with an increased activation of Smad3 in Hp-infected gastric mucosa.

Surprisingly, however, the gastric biopsies collected from Hp-positive patients exhibited reduced levels of p-Smad3 compared with controls, as shown in figure 3, A. In fact the analysis of active/non-active Smad3 ratio shows a significant decrease in the Hp-positive patients compared with controls (P <0,001) (figure 3, panel B). The decrease of

Smad3 activation, as assessed by the level of its phosphorylation, during Hp Infection was also evident in gastric LPMCs (figure 3, panel C)

The TGF-β1/Smad signaling system is characterised by the existence of an inhibitory mechanism that mainly involves Smad7 (Nakao et al., 1997).

In order to verify whether the reduced activation of Smad3 during Hp Infection was associated with changes in Smad7 expression, the proteins extracted from gastric biopsies and used for the analysis of phosphorylated Smad3 were then subsequently analysed for the content of Smad7. Smad7 protein expression was increased in all patients with Hp infection- associated gastritis compared with Hp-negative patients (figure 4, panel A). In fact, Hp-positive patients have a mean value of Smad7/β-actin ratio of 0.652 densitometry arbitrary units (range 0.4-0.98) that is significantly higher than that found in Hp-negative patients (mean 0.125; range 0.001-0.3) ($P < 0.001$), (figure 4, panel B).

The enhanced expression of Smad7 was also observed in the LPMC samples collected from patients with Hp Infection, as shown in figure 4, panel C.

In order to show that Hp colonisation of gastric mucosa is closely responsible for Smad7 induction, the Smad7 expression was analysed in gastric biopsies of 5 Hp-positive patients before and after a successful eradicating therapy. Hp eradication results in a reduction of Smad7 expression (panel D).

In addition it is provided evidence suggesting a mechanism by which Smad7 can be induced in the Hp-infected gastric mucosa.

Although *Helicobacter pylori* is a non-invasive bacterium, there are several evidences regarding to the ability of bacterium to release factors that penetrate into the mucosa and stimulate mucosal inflammatory cells (Ernst et al., 2000, Israel et al., 2001).

The evidence that Smad7 expression is up regulated at the lamina propria level in Hp-colonised mucosa raised the hypothesis that induction of Smad7 could be due to molecules induced by Hp infection within the lamina propria. In order to verify such a hypothesis the gastric biopsies taken from Hp-negative patients were cultured in presence of a filtrate culture broth derived from a Hp wild-type strain or cultured in presence of rh-IFN-γ and assessed for Smad7 expression.

As shown in figure 5, panel A, the stimulation of normal gastric biopsies with IFN-γ, but not with Hp culture broth increases Smad7. It is

noteworthy that the Hp culure broth filtrate induces IL-8 in AGS cells, clearly showing the functional activity of the broth (figure 5, panel B).

Together these results suggest that IFN-γ, which is produced in excess during the Hp associated Inflammatory response, induces Smad7. In order to verify this, gastric biopsies taken from Hp-positive patients were cultured in the presence of neutralising IFN-γ antibodies and then analysed for Smad7 content. As shown in figure 5, panel C, the neutralising IFN-γ but not the control antibody reduced significantly Smad7 expression.

It is known that IFN-γ acts by activating intracellular signals linked to the STAT1 pathway (Horvath et al., 1997) and several previous studies have shown that IFN-γ-induced STAT1 activation can be associated with Smad7 induction in monocyte cell lines (Ulla et al., 1999).

On the basis of these observations, it has been tested the effect of TB42, a STAT1 inhibitor, on the expression of Smad7 induced in gastric biopsies by IFN-γ. Figure 6 shows the TB42-mediated inhibition of STAT1 and Smad7 induced by IFN-γ.

In the present study it has also been observed that Smad7 inhibition restores the TGF-β1 mediated signalling and that this results in a decreased IFN-γ and T-bet expression in Hp colonized gastric biopsies.

As the role of Smad7 is to inhibit or to destroy the TGF-β1-mediated signal cascade and a defective activity of TGF-β1 has been associated with high levels of Smad7 (Nakao et al., 1997) it has been hypothesized that Smad7 inhibition in Hp infected biopsies could allow the endogenous TGF-β1 to dampen the local inflammatory response.

The Hp related inflammation is associated with exaggerated production of Th1 cytokines, whose expression could depend on specific transcription factors such as T-bet (Ernst et al., 2000, Israel et al., 2001, Szabo et al., 2000).

Using an *ex vivo* organ culture of gastric biopsies collected from Hp-positive patients, it has been determined whether inhibition of Smad7 could reduce IFN-γ and T-bet in tissue. After 24 hours of culture in medium, supplemented or not with sense oligonucleotides for Smad7, Smad7 protein was expressed at high levels and this was associated with a reduced phosphorylation of Smad3 and increased expression of IFN-γ and T-bet.

In contrast, the inhibition of Smad7 by antisense oligonucleotide resulted in increased p-Smad3, and consensual decrease of IFN-γ and T-bet in tissue level, as shown in panels A and B of figure 7.

In order to confirm these results, IFN-γ protein was also analysed in the organ cultures supernatants. After 24 hours of culture in medium, Hp colonised biopsies release IFN-γ in the culture supernatants and the concentration levels of the protein were not affected by the addition of Smad7 sense oligonucleotide, as shown in figure 7, panel C. According to Western blotting results, the Smad7 inhibition on the contrary caused a marked reduction of levels of IFN-γ secreted in the culture supernatant.

The results reported in the previous example show that the colonization of the stomach by *Helicobacter pylori* involves a drastic reduction in Smad3 phosphorylation, a crucial step in the TGF-β1-mediated signal transduction mechanism, despite the elevated expression of TGF-β1 receptors and synthesis of active TGF-β1.

The failure in TGF-β1 factor signal pathway is associated with increased expression of Smad7, an inhibitory protein of the Smad3 phosphorylation process mediated by TGF-β1. In addition it has been shown that the restoration of the TGF-β1 activity, by Smad7 antisense oligonucleotide, causes a marked reduction in the synthesis of inflammatory molecules secreted by Th1 cells, which seem to have a key role in the activation of other Inflammatory pathways such as the NF-kB.

EXAMPLE 2: *Role of Smad7 in sustaining the increased activity of NF-kB in chronic intestinal Inflammatory diseases (IBD).*

MATERIALS AND METHODS

Patients and samples

The mucosal samples were taken from 11 CD patients with moderate to severe activity. The primary site of the lesions was the colon in 9 patients, while in 2 patients both the colon and terminal ileum were involved. Eight patients were treated with mesalazine and/or with antibiotics and the remaining 3 with corticosteroids.

The indication for surgery was the unresponsiveness to pharmacological treatment. In addition, mucosal samples were taken from 3 patients with UC undergoing colectomy, due to unresponsiveness to medical therapy. As controls, 14 patients undergoing colectomy for

colonic cancer were recruited. In each of these 14 patients the mucosal samples were collected from macroscopically and histologically unaffected areas. The approval was obtained by the local ethical committee.

Isolation and culture of mononuclear cells of the intestinal lamina propria

LPMC were prepared as described previously (Monteleone et al., 1997) and an aliquot was immediately used for extracting cytosolic and nuclear proteins.

The remaining LPMC were resuspended in RPMI 1640 (Sigma Chemical Aldrich, Dorset, UK) supplemented with HL-1 (Biowhittaker Wokingham, UK), and cultured in the presence or absence of TGF-β1 (final concentrations ranging from 1 to 5 ng/ml, Sigma) for 1-28 hours and then stimulated or not with TNF-α (5 ng/ml; Peprotech EC Ltd, London, UK) for the indicated times.

In other experiments, IBD LPMC were resuspended in RPMI 1640 supplemented with HL-1 and cultured in the presence or absence of a Smad7 antisense oligonucleotide or control (sense) oligonucleotide (both used at a final concentration of 2 μg/ml) for 24 hours in absence of lipofectamine.

The Smad7 anti-sense and sense oligonucleotide sequences have previously been described (Monteleone et al., 2001). Briefly, phosphorothioate single strand oligonucleotides matching the region 107-128 (5'-GCTGCGGGGAGAAGGG GCGAC-3 ') of the human Smad7 coding sequence were synthesised in sense and antisense orientation.

Since the phosphorylated anti-sense oligonucleotide contains two CpG motifs that could have immunostimulatory activity, the cytosine in dinucleotides was replaced with a 5-methylcytosine. In addition phosphodiester bonds were replaced with methylphosphonates at the ends of the oligonucleotide to increase the stability of molecule. In four separate experiments, IBD LPMC were treated with the antisense oligonucleotide in the presence or absence of a neutralising TGF-β1 antibody (5 μg/ml; R&D Systems, Abingdon, UK). After 24 hours, an aliquot of LPMC was used to extract the proteins for Smad7 analysis. To assess the effect of IkBα, the cells were washed and resuspended in RPMI 1640 plus HL-1 and cultured in the presence or absence of TGF-β1 (5 ng/ml) for 7-14 hours and then stimulated with TNF-α.

RNA extraction, preparation of complementary DNA (cDNA) and RT-PCR

Total RNA, preparation of cDNA and RT-PCR were carried out as previously described (Monteleone et al., 1997). PCR primers were the followings:

IL-8, FWD, 5'-TGCAGCTCTGTGTGAAGG-3' (SEQ ID No 4);
REV, 5'-ATTGCATCTGGCAACCCTAC-3' (SEQ ID No 5);

IkBα, FWD: 5'-ATCACCMCCAGCCAGAMT-3' (SEQ ID No 6);
REV: 5'-GCACCCMGGACACCMAAG-3' (SEQ ID No 7);

α-actin, FWD: 5'-GGC ACC ACA CCT TCT ACA-3' (SEQ ID No 8);
REV: 5'-CAG GTC TTT GCG GAT GTC-3' (SEQ IDs No 9).

RT-PCR products were electrophoresed on 1% agarose gel containing 0.3 μg/ml of ethidium bromide.

Electrophoretic mobility shift assay (EMSA)

Nuclear protein-DNA birding studies were carried out for 20 minutes at room temperature in a 20 ml reaction volume containing 10 mM Tris-HCl, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5mM $MgCl_2$, 1 μg Poly (dI-dC), (Sigma) 50 fmol biotin labelled oligonucleotides containing probe and 10 μg of nuclear proteins.

The DNA probes were prepared by annealing the two consensus oligonucleotides (FWD, 5': -AGTTGAGGGGACTTTCCCAGG-3' SEQ ID No 10, REV, 5':-CGGACCCTTTCAGGGGAGTTGA-3', SEQ ID No 11), which were labelled at 3' end with biotin using a commercially available kit (Pierce, Rockford, IL, U.S.A.). The binding specificity was confirmed by incubating the nuclear proteins with samples unlabelled NF-kB probe or with unlabelled oligonucleotides of the IL2 gene (IL2G), (5':ACMCGCGTGAGCTCTCTAGAAAGCATCATCTCAACAC-TMCTTGATMTTMGTGCCTCGAGCACA-3', SEQ ID No 12) in a 100-fold molar excess in order to saturate the binding.

In antibody neutralisation experiments, a monoclonal antihuman NF-kB/p65 or p50 monoclonal (Saint Cruz Biotechnology, Saint Cruz, CA, USA) or a control anti-isotype IgG antibody (Dako Ltd) (both used at the concentration of 2.5 μg) were incubated with nuclear proteins for 45 minutes prior to adding the probes. A 6% non-denaturing polyacrylamide gel was used for the electrophoretic separation. After blotting on nitrocellulose membrane, the labelled oiligonucleotides were detected with a chemiluminescence EMSA kit (Pierce).

Protein extraction and Western Blot analysis

LPMC were homogenised and cytosolic extracts collected in buffer A containing 10 mM Hepes (pH 7,9), 10 mM KCl, 0,1 mM EDTA and 0,2 mM EGTA. Nuclear extracts were prepared by dissolution of the remaining nuclei in buffer C containing 20 mM Hepes (pH 7,9), 0,4 M NaCl, 1 mM EDTA, 1 mM EGTA and 10% glycerol. Both buffers were supplemented with 1 mM dithiothreitol (DTT), 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenilmethansulphonyl fluoride (Sigma).

For the detection of cytosolic and nuclear NF-kB/p65, 10 μg/sample of nuclear or cytosolic proteins were separated on 10% SDS-PAGE gel. p65 was detected by using a rabbit anti human NF-kB/p65 antibody (final dilution 1:500) (Saint Cruz Biotechnoìogy) and subsequently a goat antibody IgG, conjugated to horseradish-peroxidase (DAKO, Cambridgeshire, U.K.) (final dilution 1:2500). The reaction was performed using an ECL kit (Amersham Pharmaceuticals, Amersham, U.K.). In order to ascertain equivalent loading and transfer of proteins, with ponceau 5 (Sigma) staining was performed .

After detection of p65protein, blots was stripped and analysed for the content of histone1 using a mouse anti human-histone1 monoclonal antibody (final dilution 1:300, Saint Cruz Biotechnology) and subsequently a goat anti-mouse antibody goat antibody conjugated to horseradish-peroxidase (dilution 1:1500, Dako Ltd).

IkBα expression was detected by Western blotting using a rabbit anti human IkBα antibody (final dilution 1:500, Saint Cruz Biotechnology). Blots were stripped and reincubated with the antibody for β-actin as previously specified (Monteleone et al., 2001).

Smad7 was analysed using a specific rabbit anti human Smad7 antibody (final dilution 1:400, Saint Cruz Biotechnoìogy, Inc.). Goat anti-rabbit antibody conjugated to horseradish peroxidase (dilution 1:20,000, Dako Ltd) was used for the detection of primary antibody binding and immunoreactivity was visualised with a chemiluminescence kit (Pierce).

Plasmid DNA

All the plasmid DNA were prepared using a commercial extraction and isolation kit (Maxiprep, Qiagen, UK). A luciferase reporter vector containing the nucleotides from -332 to +35 of the human IkB-α gene promoter was provided by Professor Ron Hay (St Andrews, UK). The control Renila luciferase vector pRLTK was purchased from Promega (Southampton, UK).

Transfection analysis and reporter gene assay

Primary foetal gut fibroblasts (Bajaj-Eliot et al., 1998) were transfected using the non-liposomal Effectene protocol (Qiagen) according to the manufacturer's instructions.

Luciferase assays were performed using a dual luciferase kit (Promega).

The expression of firefly luciferase (pIkBα-Luc) under the control of the IkBα promoter was normalised for differences in expression efficiency, by measuring the activity of a co-transfected Renilla luciferase vector (pRLTK).

Quantitative analysis

Western blots or PCR gels were scanned on a densitometer and the values expressed in arbitrary units (u.a.). The differences in the intensity of the bands were compared using the Mann-Whitney U test.

RESULTS

It is shown that TGF-β1 inhibits NF-kB activation in normal LPMC. Stimulation of normal LPMCs with TNF-α alone results in a marked nuclear translocation of p65 (figure 8, panel A). Preincubation of LPMC with TGF-β1 prevents the TNF-α-induced p65 nuclear translocation, with no change in the content of cytosolic NF-kB (figure 8, panel B).

Stimulation of normal LPMC with TNF-α also increases the NF-kB binding activity, as shown in figure 9. The incubation with an excess of oligonucleotide spanning a binding site of the IL-2 gene does not antagonize the NF-kB binding, while the competition with an excess of un-labelled NF-kB oligonucleotide led to inhibition of the binding activity. The analysis of the subunit composition of the DNA complex by supershift assay shows that TNF-α increases both p65/p50 heterodimers and p50 homodimers (figure 9). Preincubation of LPMC with TGF-β1 reduces TNFα-induced NF-kB DNA complexes with a more marked effect on the p65/p50 heterodimers (figure 9).

IL-8 transcription is induced by NF-kB and IL-8 levels are increased in IBD (Bows et al., 1998).

Since it has been established that TNF-α up regulates IL-8 transcription through a NF-kB-dependent mechanism (Baldwin et al., 1996), the authors of the present invention have determined whether TGF-β1 inhibited IL-8 gene expression. As expected LPMCs treated with TNF-α show a marked increase in IL-8 RNA transcripts, as shown in figure 10, panel A. However the TNF-α-induced IL-8 transcripts were

approximately reduced of 95% by pre-treatment with TGFβ1 (figure 10, panel B).

The study has further underlined that TGF-β1 inhibits the NF-kB/p65 nuclear translocation and induces IkBα in normal LPMC.

In most cells, NF-kB proteins are maintained as inactive protein complexes in the cytoplasm by the inhibitory proteins IkBs, among which IkBα represents a prototype. Activation of NF-kB requires IkB phosphorylation which is then followed by its ubiquitination and degradation. This allows the free NF-kB dimers to translocate into the nucleus (Baldwin et al., 1996).

Therefore, one potential mechanism by which TGF-β1 suppresses NF-kB activation could be related to its ability to prevent p65nuclear translocation.

To this end, IkBα protein expression was analysed in cytosolic extracts isolated from LPMCs pre-incubated with TGF-β1 for 7 hours and then treated with TNF-α for various times. In un-treated LPMC, the cytoplasmic IkBα signal disappears almost completely after 20 minutes of treatment with TNF-α and returns to the original level after 40 minutes of stimulation (figure 11, panel A).

Pre-treatment of LPMC with TGFβ1 for 7 hours results in an increased IkBα expression, so that the following stimulation with TNFα does not decrease IkBα, (figure 11, panel A. The key change in LPMCs pre-treated with TGF-β1 (compared to cells treated with medium) prior to TNF-α stimulation was the substantial increase in IkBα/β-actin ratio at all time points after treatment (figure 11, panel B).

In order to confirm that TGFβ1 results in an IkBα increase, cytosolic extracts were prepared from normal LPMC treated for various time points and analysed by Western blotting. TGF-β1 treatment results in an increased expression of IkBα protein for up to 28 hours, as can be observed in the panel C of figure 11.

TGF-β1 also increases IkBα transcripts in normal LPMCs, as shown in the panel D of figure 11.

Subsequently primary gut myofibroblasts were transfected with a construct containing the luciferase reporter gene under the transcriptional control of the human IkBα promoter (pIkBα-Luc) or with a control vector pRLTK. 16 hours after transfection, cells were incubated with TGF-β1 for 2, 4 or 24 hours. TGF-β1 increases the transcriptional

activity by approximately about 2,5 and 3,5-folds after 2 and 4 hours, as shown in figure 12.

It has also been shown that IBD LPMC have high NF-kB/p65 nuclear accumulation and low IkBα cytosolic content.

To further prove that, in IBD, LPMC show an enhanced NF-kB/p65 activation (Neurath et al., 1998, Schreiber et al., 1998, Neurath et al., 1996), the nuclear proteins were isolated both from normal and IBD LPMC and analysed by Western blotting. A strong immunoreactivity for NF-kB/p65 was seen in nuclear extracts of IBD LPMCs in comparison to control samples (figure 13, panel A). The analysis of the p65/histone-1 ratio shows a mean value of 0,91 densitometric arbitrary units (range 0.54-1.23) in patients with IBD and 0.26 in controls (range 0.08-0.4) ($p < 0.02$) (figure 13).

Significantly, the high p65 nuclear accumulation documented in IBD LPMC was associated with a reduced cytoplasmic IkBα expression as compared with normal LPMC (figure 13, panel B). Furthermore, two bands, corresponding to the basal and phosphorylated form of IkBα, were identified in the samples of LPMC affected by IBD, as shown in figure 13, panel B.

The authors of the present invention have further addressed the possibility that TGF-β1 is able to inhibit NF-kB activation in cells from IBD patients. The already high nuclear level of NF-kB/p65 increases modestly after treatment with TNFα (figure 14, panel A). However, pre-treatment with TGF-β1 has not effect. Likewise TGFβ1 does not reduce the DNA binding activity in extracts of IBD LPMC, as shown in figure 14, panel B.

It has been observed that Smad7 inhibition in IBD LPMC allows TGF-β1 to down regulate NF-kB activation.

In both CD and UC LPMC, there is a reduced activity of TGF-β1-mediated signal cascade due to high Smad7 expression levels (Monteleone et al., 2001).

Thus the sustained activation of NF-kB documented in IBD LPMCs could result from a defective TGF-β1-mediated signal transmission. To address this issue, IBD LPMCs were treated with Smad7 sense and antisense oligonucleotides and then analysed for both nuclear NF-kB/p65 and cytosolic IkBα. The treatment with Smad7 antisense inhibits the Smad7 expression, as shown in figure 15, panel A, clearly confirming the efficiency of oligonucleotides transfection.

Both untreated or treated with Smad7 sense oligonucleotide LPMC show high levels of nuclear p65 and low cytosolic levels of IkBα, as shown in figure 15, panels B and C.

However in antisense treated LPMCs there was a marked increase in cytosolic levels of IkBα and a reduced nuclear p65 immunoreactivity as can be observed in figure 15, panels B and C, which was prevented by the addition of a TGF-1-neutralizing antibody, suggesting that the Smad7 inhibition allows the endogenous TGF-β1 to inhibit NF-kB.

The addition of a control IgG to the antisense treated IBD LPMC cultures does not result reduce cytoplasmic IkBα or increase nuclear p65.

The evaluation of the NF-kB DNA binding activity was used to verify, independently, the effects of Smad7 antisense oligonucleotide .

The IBD LPMCs pre-treated with medium or with sense oligonucleotide and then cultured in medium alone show high binding NF-kB activity, as shown in figure 16, which was slightly increased following the treatment with TNF-α and only modestly decreased by pre treatment with TGF-β1. In contrast, IBD LPMC treated with Smad7 antisense oligonucleotide show a markedly decrease in NF-KB binding activity. In these cells, stimulation with TNF-α results in a high NF-kB binding which was prevented by incubation with TGF-β1. as shown in figure 16.

Within this experimentation the authors of the present invention show that TGF-β1 is a potent negative regulator of the transcription factor NF-kB in the human intestine. Initially it has been shown that the pre-treatment of normal LPMCs with TGF-β1 results in the inhibition of TNFα-induced nuclear translocation of p65 and this was associated with TGF-β1-mediated induction of elevated IkBα levels. Both the NF-kB binding activity and expression of the NF-kB-dependent IL-8 gene, mediated by TNF-α, were dramatically inhibited by preincubation of LPMCs with TGF-β1. In marked contrast, TGFβ1 was unable to inhibit NF-kB activation in LPMCs from patients with IBD.

Although this finding could partly rely on the different state of activation of LPMC in IBD and normal mucosa (Fiocchi et al.,1998), it would seem that the high Smad7 levels seen in the inflamed mucosa of by IBD patients (Monteleone et al., 2001) can well contribute. Indeed, decreasing Smad7 levels with an antisense oligonucleotide restored the

positive effect of endogenous TGF-β1 on IkB-α expression which eventually led to a reduced NF-kB accumulation in the nucleus.

EXAMPLE 3: *Constitutive expression of Smad7 in gastric and colonic epithelial cancer cells.*

MATERIALS AND METHODS

All the cell lines were purchased from Sigma (Milan, Italy) and maintained in culture in 75 cm^2 plates with DMEM (HT-29, HT-115 and Caco2) or DMEM-F12 (the remaining lines) supplemented with 10% FBS.

After the achievement of a 70-80% confluence, cells were detached from the plate, by treatment with trypsin-EDTA solution, and used for RNA extraction, cDNA preparation and then RT-PCR. Such methods were performed using the protocols described in the preceding paragraphs of this disclosure.

The specificity of PCR products for Smad7 was confirmed by sequencing the product.

Numerous evidences support the link between mucosal inflammation and cancer development. For example, an increased risk of cancer is observed in patients with Hp-related gastritis and IBD.

Moreover, it is well known that loss of TGF-β1 activity plays a role of primary importance in the development and progression of tumours of digestive tube.

On the basis of these considerations it is conceivable that induction of Smad7 and the consensual loss of TGF-β1 activity may have a pathogenetic role in human tumour pathology.

In order to delineate such sequence the authors of the present invention provide preliminary evidences showing a constitutive expression of Smad7 in epithelial cell lines generated from human gastric and colonic adenocarcinoma (figure 17).

BIBLIOGRAPHY

- Li Q, et al., 2002. Nat. Rev. Immunol. 2:725-734.
- Zingarelli B.et al, 2003. Crit. Care Med. 31: S105-111.
- Sasaki et al, 2001. Clin Cancer Res. 7: 4136-4142.
- Yu HG et al., 2003. Oncology. 65: 37-45.
- Maeda, S, Akanuma, M.., Mitsuno.Y, Hirata, Y, Ogura, K, Yoshida, H, Shiratori, Y., Ornata, M. 2001. J. Of biol. Chem. 276:44856-44864.
- Maeda, S., Akanuma, M.., Mitsuno.Y, Mirata, Y., Ogura, K., Yoshida, H, Yamaji, Y., Shiratori, Y, Ornata, M. 2000. Gastroenterology 2000. 119:97-108.
- Strober W, Fuss, I.J, and Blumberg, R. 2002. Annu Rev Immunol. 20: 495-549.
- Han S.H, Yea, S.S, Jeon, Y.J., Yang, K.H. and Kaminsky, N.E, 1998. J Pharmacol Exp Ther. 287; 1105-12.
- Monteleone, G, Kumberova, A., Croft, N.M., McKenzie, C., Steer, H. W., and MacDonald, T.T. 1991. J Clin Invest 108:601-609.
- Hayashi, H, Abdollah, S., Qiu, **Y.,** Cai, J., Xu, **Y.Y,** Grinnell, B.W, Richardson, M.A., Topper, J.N., Gimbrone Jr, M.A., Wrana, J.L, and Falb, D. 1997. Cell 89: 1165-1173.
- Ernst, P. B, and Gold, B. D. 2000. Annu. Rev. Microbiol. 54:615-40.
- Israel, D. A, and Peek, R. M. 2001. Aliment Pharmacol Ther 15:1271-1290.
- Chu, S.H, Kim, H., Seo, J.Y., Lim, J.W, Mukaida, N, and Kim, K.H. 2003. Dig Dis Sci 48:257-265.
- Bhattacharyya, A., Pathak, S., Datta, S., Chattopadhyay, S., Basu, J., and Kundu, M. 2002. Biochem J 368:121-129.
- Cover, T.L, Krishna, U.S., Israel, D.A., and Peek, R.M. Jr. 2003. Cancer Res 63:951-957.
- D'Elios, M.M,, Manghetti, M., De Carli, M., Costa, F., Baldari, C.T., Burroni, D., Telford, J. L., Romagnani, S., and Del Prete, G. 1997. J.Immunol 158:962-967.
- Smythies, L.E., Waites, K.B., Lindsey, J.R., Harris, P.R., Chiara, P., and Smith, P.D, 2000. J Immunol 165:1022-1029.

- Eaton, K.A., Mefford, M., and Thevenot, T. 2001. J Immunol 166:7456-7461.
- Letterio, J.J., and Roberts, A.B. 1998. Annu Rev Immunol 16:137-161.
- Wahl, S.M. 1994. J. Exp. Med. 180:1587-1590.
- Hahm, K.-B, Lee, K.M., Kim, Y. B., Hong, W. S., Lee, W. H., Han, S. U, Kim, M. W., Ahn, B. O., Oh, T. Y., Lee, M. H., Green, J., and Kim, S. J. 2002. Aliment Pharmacol Ther 16 (Suppl. 2): 115-127.
- Fais, S., Maiuri, L, Pallone, F., De Vincenzi, M., De Ritis, G., Troncone, R., and Auricchio, S. 1992. Gut 33:472-475.
- Ricci, V., Ciacci, C., Zarrilli, R-, Sommi, P., Tummuru, M. K-, Del Vecchio Bianco, C., Bruni, C. B., Cover, T. L., Blaser, M. J., and Romano, M. 1996. IFNect Immun 64: 2829-2833.
- Monteleone, G., Biancone, L, Marasco, R., Morrone, G., Marasco, O., Luzza, F., and Pallone. F: 1997. Gastroenterology 112: 1169-1174.
- Barranco, S. C., Townsend Jr., C. M., Casartelli, C., Macik, B. G., Burger, N. L., Boerwinkie, W. R., and Gourley, W. K. 1983. Cancer Res 43:1703-1709.
- Yang, **X.,** Letterio, J.J., Lechleider, R. J., Chen, L., Hayman, R., Gu, K, Roberts, A. B., and Deng, C. 1999. EMBO J 18:1280-1291.
- Heldin, C.-H., Kohei, M., and ten Dijke, P. 1997. Nature. 390:465-471.
- Derynck, R., Zhang, Y., and Feng, X.-H. 1998. Cell. 95:737-740.
- Nakao, A,, Afrakhte, M., Moren, A., Nakayama, T., Christian, J.L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N. E., Heldin, C. H., and ten Dijke, P. 1997. Nature 389: 631-635.
- Horvath, C. M., and Darnell Jr, J. E. 1997. Curr Opin Cell Biol. 9:233-239.
- Szabo, S. J., Kim, S. T., Costa, G. L, Zhang, **X.,** Fathman, C. G., and Glimcher, L. H. 2000. Cell 100: 655-669.
- Monteleone, G., Kumberova, A., Croft, N. M., McKenzie, C., Steer, H.W, and MacDonald T.T. 2001. J Clin Invest. 108: 601-609.

- Bajaj-Elliot M., Poulsom R., Pender S.L.F-, Wathen N.C., MacDonald TT1998. J Clin Invest 102:1473-1480.
- Fiocchi, C. 1998. Gastroenterology 115: 182-205. Baldwin JrA.S. 1996. Ann Rev Immunol 14: 649-681.
- Neurath, M.F., Becker C., and Barbulescu, K. 1998. Gut 43: 856-860.
- Schreiber S., Nicolaus, S., and Hampe, J. 1998. Gut 42: 477-484.
- Neurath, M.F., Petterson, S., Meyer zum Buschenfelde, K.H., and Strober, W. 1996. Nature Medicine 2: 998-1004.

CLAIMS

1. Use of Smad7 phosphorothioate antisense oligonucleotides up to 21 nucleotides in length comprising a portion of at least 10 nucleotides of the following sequence:

5'-GTXYCCCCTTCTCCCXYCAGC-3' (SEQ ID No 1)

wherein X is a nucleotide comprising a nitrogen base selected from the group consisting of cytosine, 5-methylcytosine and 2'-O-methylcytosine and wherein Y is a nucleotide comprising a nitrogen base selected from the group consisting of guanine, 5-methylguanine and 2'-O-methylguanine, provided that at least one of the nucleotides X or Y comprises a methylated nitrogen base or the sequence complementary thereof, for the preparation of a drug for the treatment of diseases mediated by the nuclear transcription factor NF-kB altered activation.

2. Use according to claim 1, wherein at least one nucleotide of the sequence is methylphosphonate.

3. Use according to claim 2, wherein at least a methylphosphonate nucleotide is placed at only one or both 3' and 5' ends or along the antisense oligonucleotidic sequence.

4. Use according to claim 2, wherein the methylphosphonate nucleotide is Y.

5. Use according to claim 2, wherein the methylphosphonate nucleotide is X.

6. Use according to claim 1, wherein at least a nucleotide of the sequence is a 2'-O-methylribonucleotide 5'-monophosphate.

7. Use according to claim 6, wherein at least a 2'-O-methylribonucleotide 5'-monophosphate is place at only one or both 3' and 5' ends or along the antisense oligonucleotidic sequence.

8. Use according to any of preceding claims, wherein 2'-deoxiribonucleotides are replaced by the corresponding ribonucleotides.

9. Use according to each of claims from 1 to 8, having the sequence:

5'-ZTXGCCCCTTCTCCCXGCAZ-3' (SEQ ID No 2)

wherein X is 5 methyl 2'-deoxicytidine 5'-monophosphate and Z is 2'-deoxiguanosine methylphosphonate.

10. Use according to claim 1, having the sequence:

5'-GTXGCCCCTTCTCCCXGCAG-3' (SEQ ID No 3)

wherein X is 5 methyl 2'-deoxicytidine 5'-monophosphate.

11. Use according to anyone of preceding claims, wherein said pathologies are selected from the group consisting of Inflammatory and tumour pathologies.

12. Use according to claim 11, wherein such Inflammatory pathologies are selected from the group consisting of *Helicobacter pylori* Infection-associated gastritis, bronchial asthma, rheumatoid arthritis, psoriasis, multiple sclerosis, Alzheimer's disease, Parkinson's disease, intestinal chronic Inflammatory diseases (IBD) and neoplastic pathologies.

13. Use according to claim 11, wherein the tumour pathology is selected from the group consisting of lymphomas and carcinomas.

14. Use according to claim 13, wherein the tumour pathology is the gastric carcinoma correlated to *Helicobacter pylori* Infection.

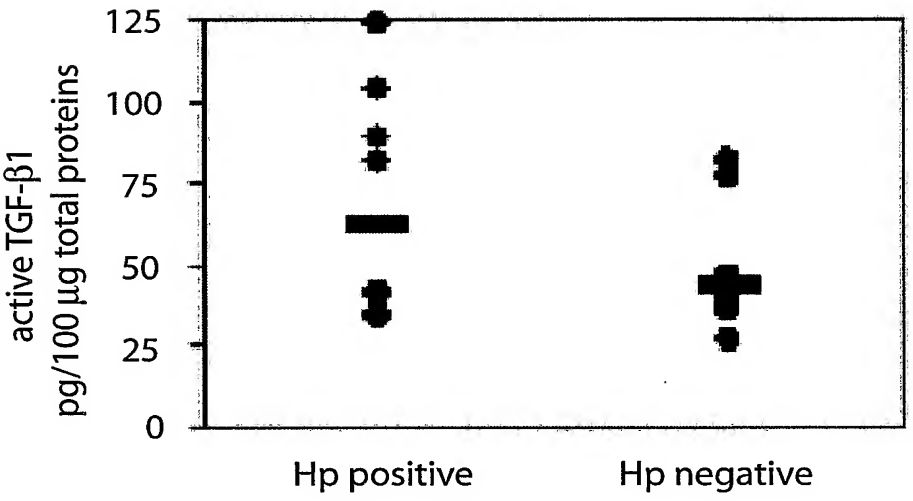
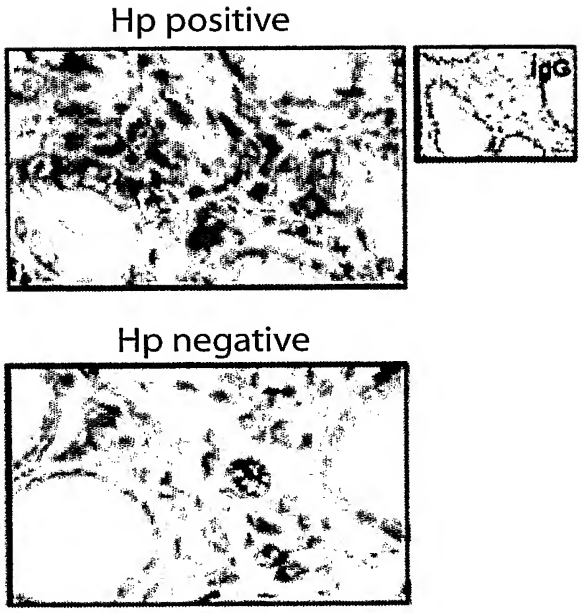

125
100
75
50
25
0
active TGF-β1
pg/100 µg total proteins
Hp positive
Hp negative

Fig. 1

A



B

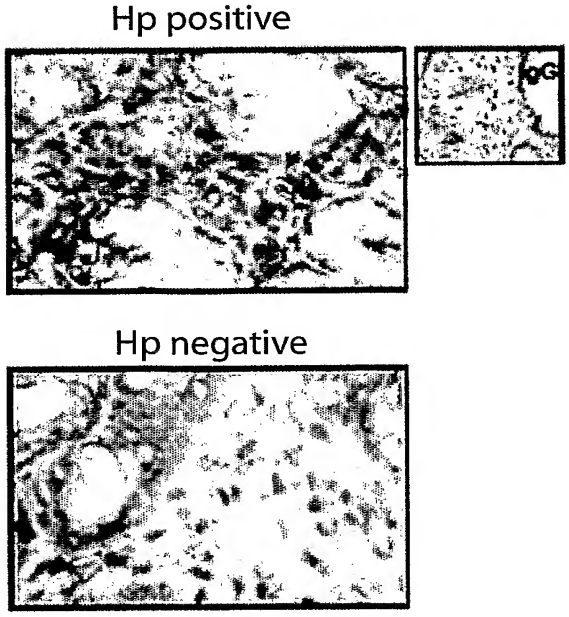


Fig. 2

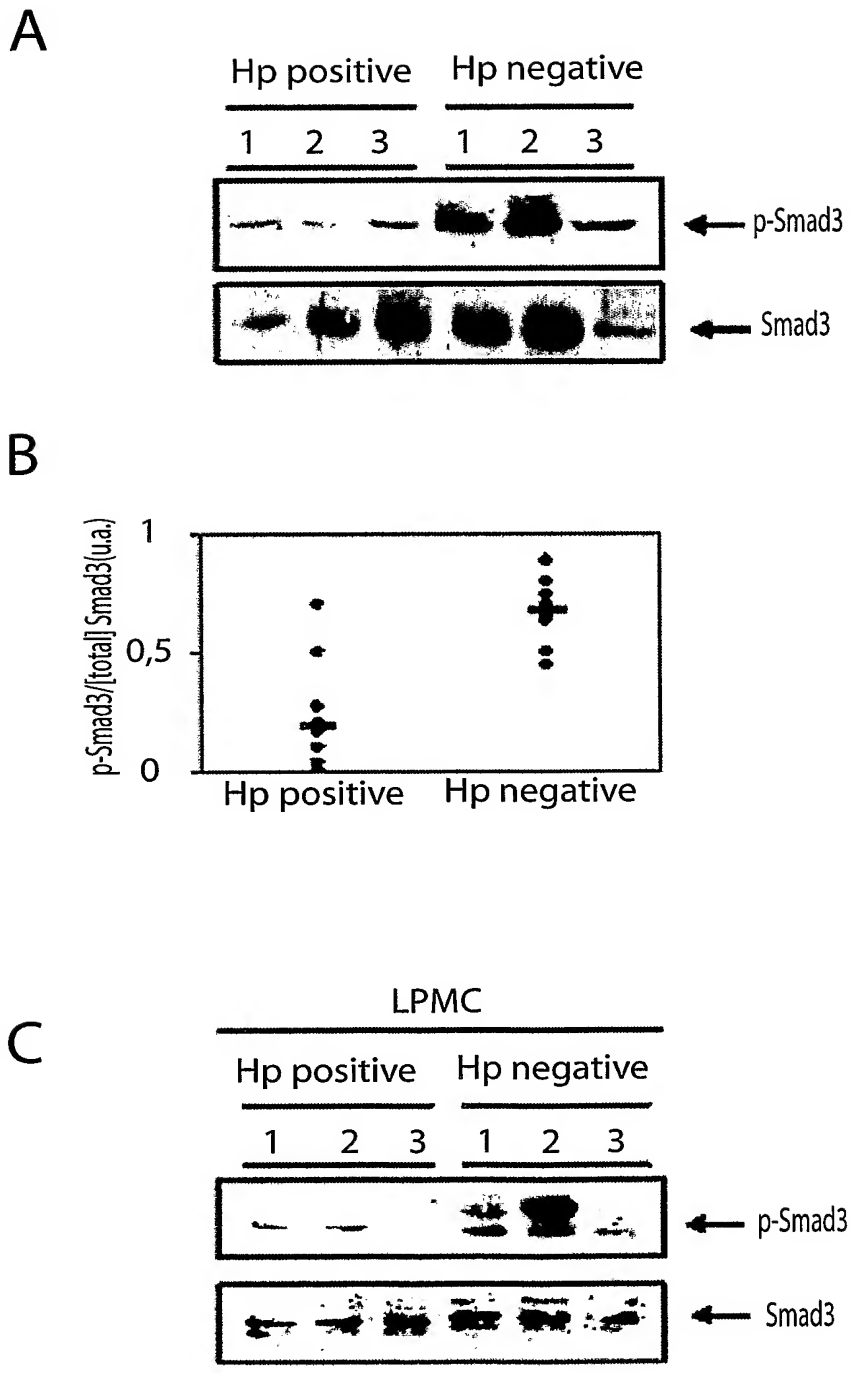

A
Hp positive
Hp negative
1 2 3 1 2 3
p-Smad3
Smad3
B
p-Smad3/[total] Smad3(u.a.)
1
0,5
0
Hp positive
Hp negative
C
LPMC
Hp positive
Hp negative
1 2 3 1 2 3
p-Smad3
Smad3

Fig. 3

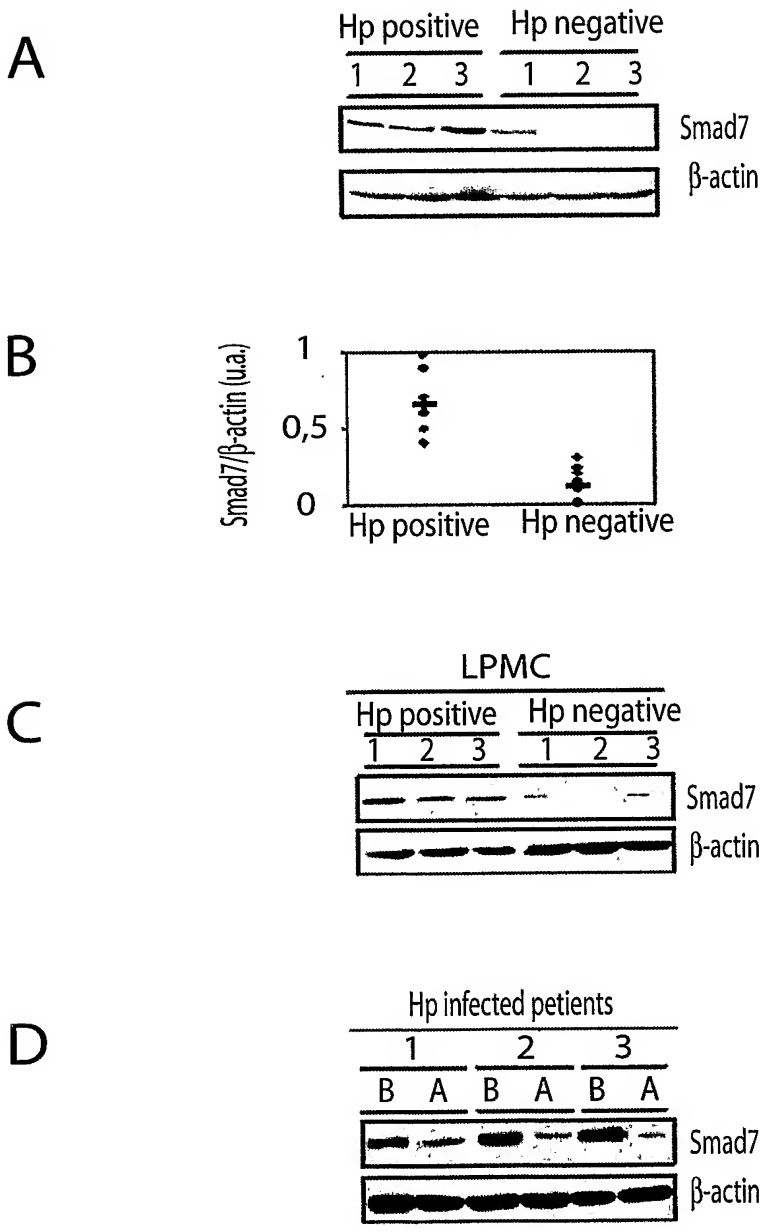

A
Hp positive
Hp negative
1 2 3 1 2 3
Smad7
β-actin
B
Smad7/β-actin (u.a.)
1
0,5
0
Hp positive
Hp negative
C
LPMC
Hp positive
Hp negative
1 2 3 1 2 3
Smad7
β-actin
D
Hp infected petients
1 2 3
B A B A B A
Smad7
β-actin

Fig. 4

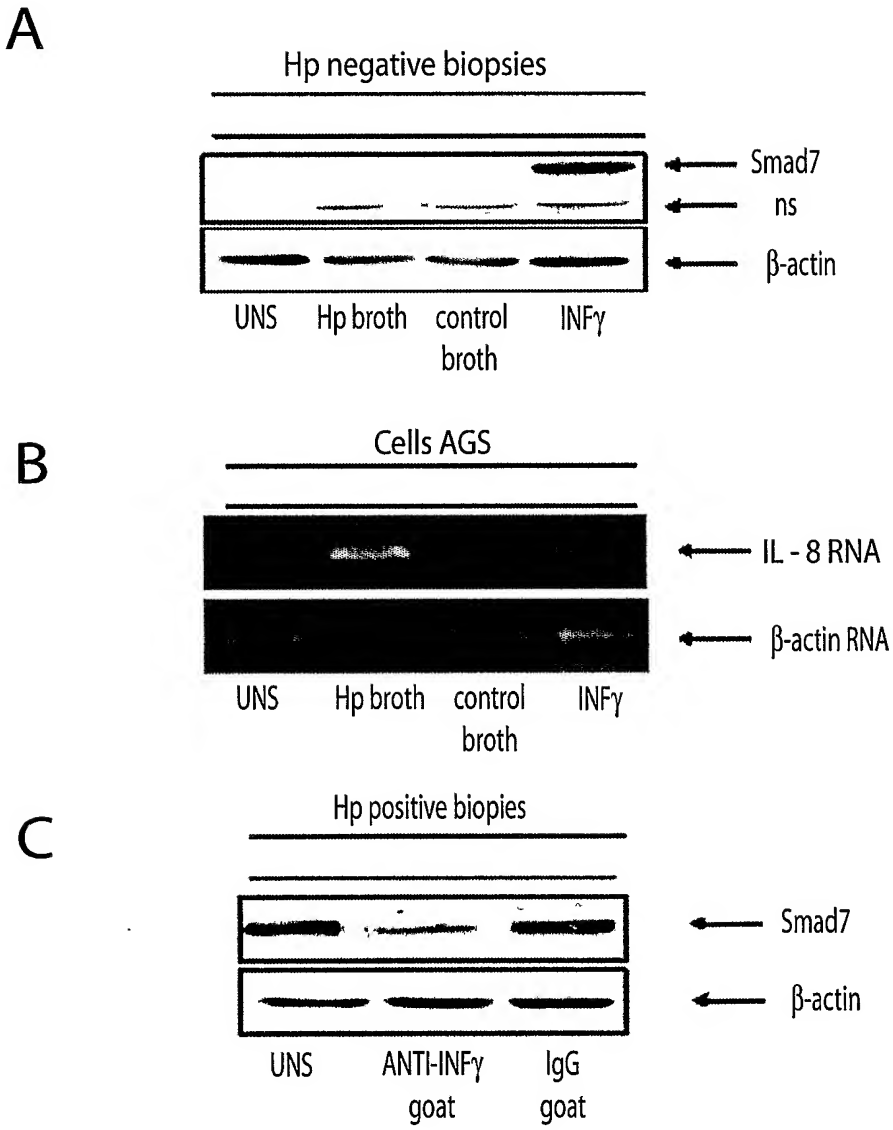

A
Hp negative biopsies
Smad7
ns
β-actin
UNS
Hp broth
control broth
INFγ
B
Cells AGS
IL - 8 RNA
β-actin RNA
UNS
Hp broth
control broth
INFγ
C
Hp positive biopies
Smad7
β-actin
UNS
ANTI-INFγ goat
IgG goat

Fig. 5

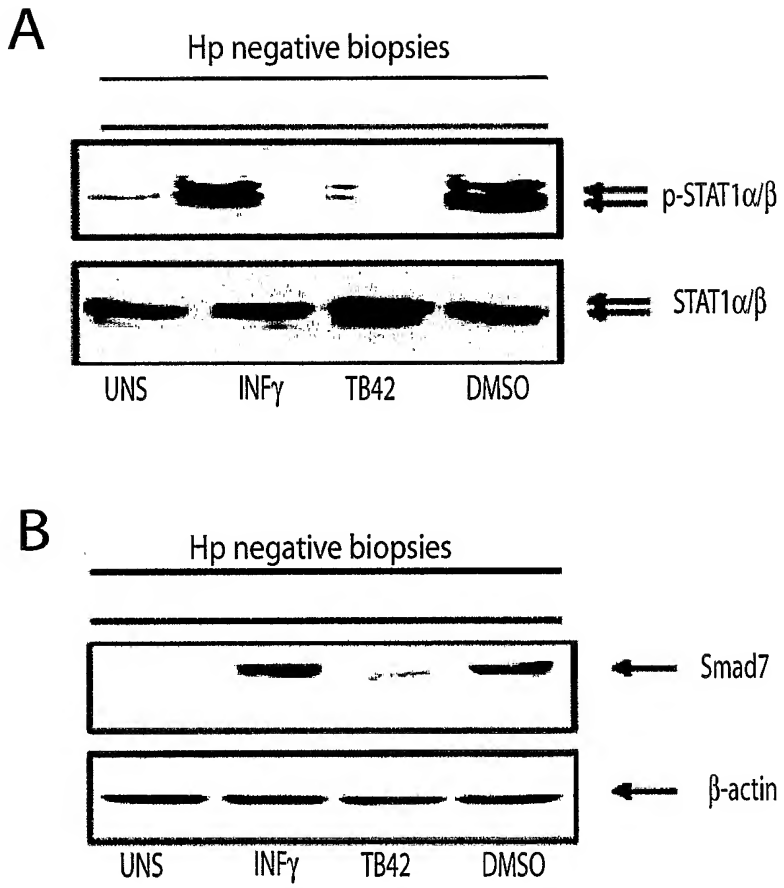

A
Hp negative biopsies
p-STAT1α/β
STAT1α/β
UNS
INFγ
TB42
DMSO
B
Hp negative biopsies
Smad7
β-actin
UNS
INFγ
TB42
DMSO

Fig. 6

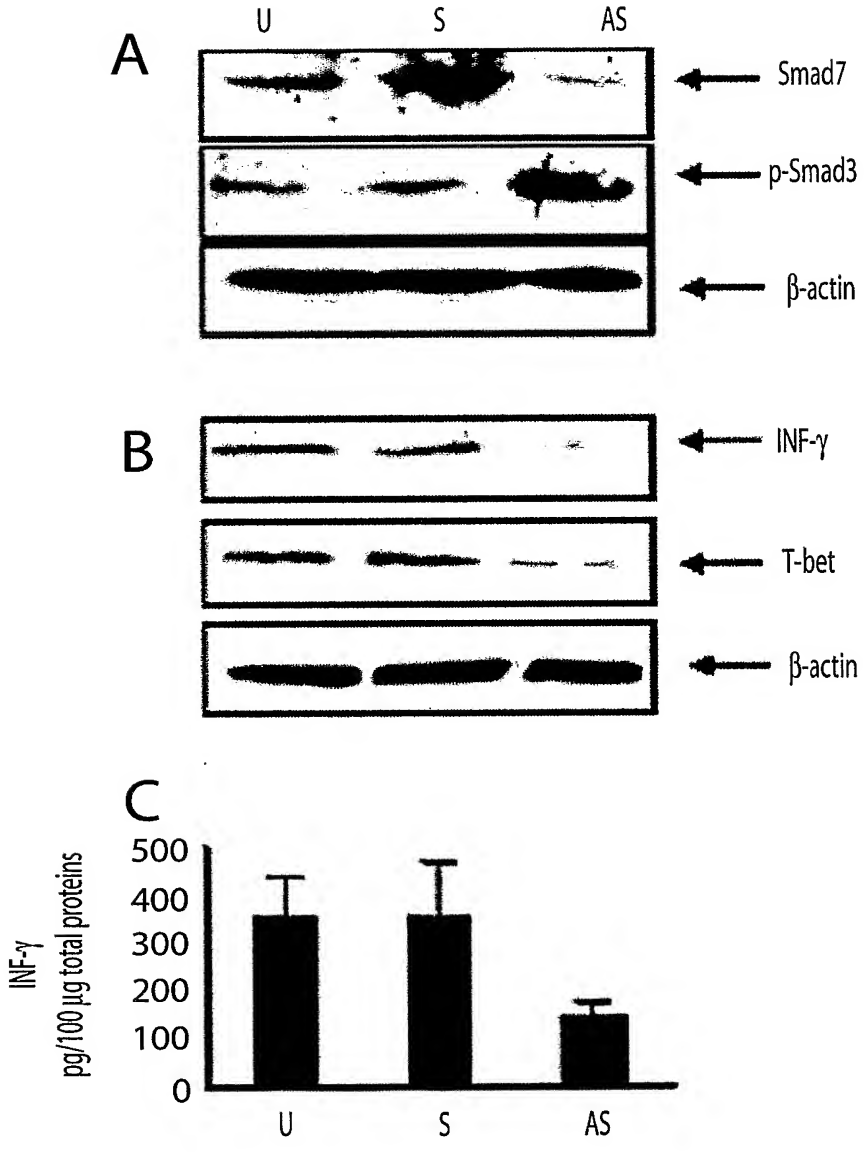
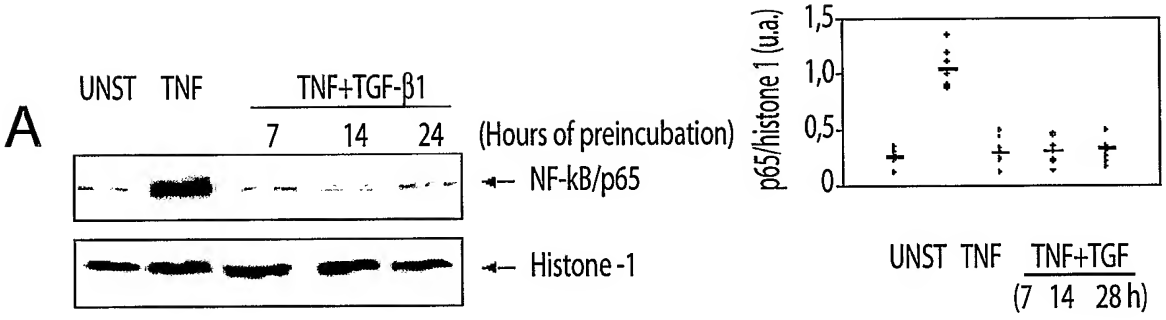

A
U
S
AS
Smad7
p-Smad3
β-actin
B
INF-γ
T-bet
β-actin
C
INF-γ
pg/100 µg total proteins
500
400
300
200
100
0
U
S
AS

Fig. 7


A
UNST TNF TNF+TGF-β1
7 14 24 (Hours of preincubation)
NF-kB/p65
Histone -1
p65/histone 1 (u.a.)
1,5
1,0
0,5
0
UNST TNF TNF+TGF
(7 14 28 h)

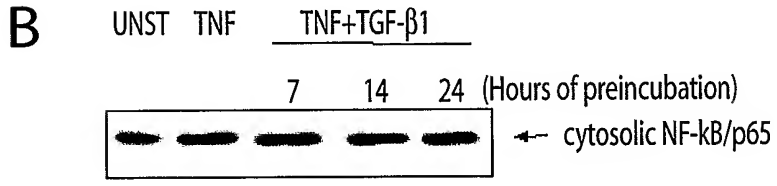

B
UNST TNF TNF+TGF-β1
7 14 24 (Hours of preincubation)
cytosolic NF-kB/p65

Fig. 8

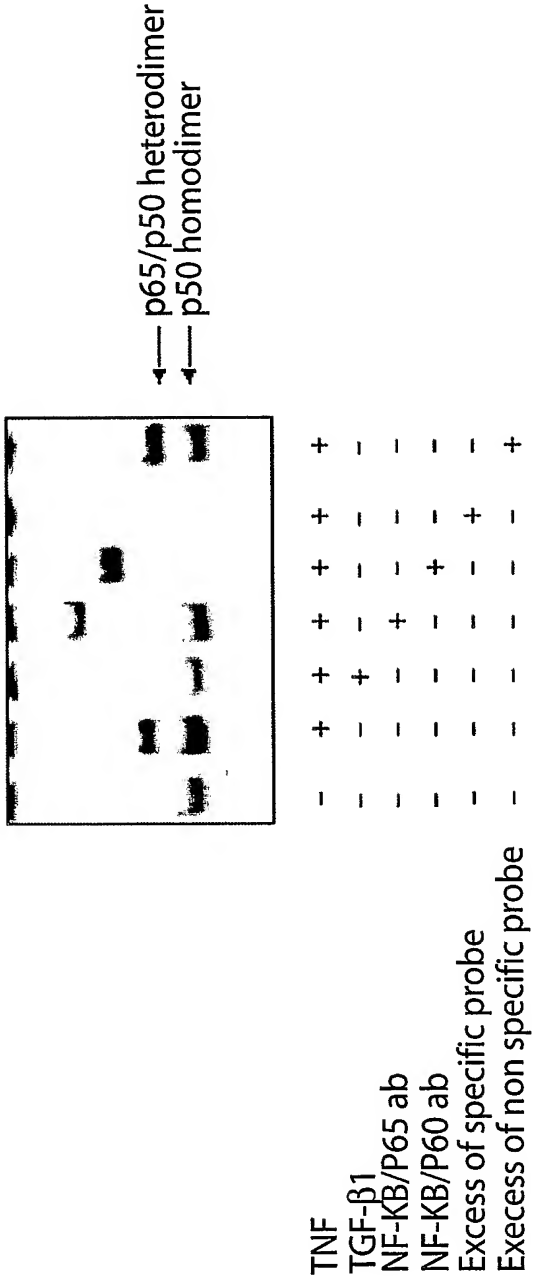

p65/p50 heterodimer
p50 homodimer
TNF
TGF-β1
NF-KB/P65 ab
NF-KB/P60 ab
Excess of specific probe
Execess of non specific probe
- + + + + + +
- - + - - - -
- - - + - - -
- - - - + - -
- - - - - + -
- - - - - - +

Fig. 9

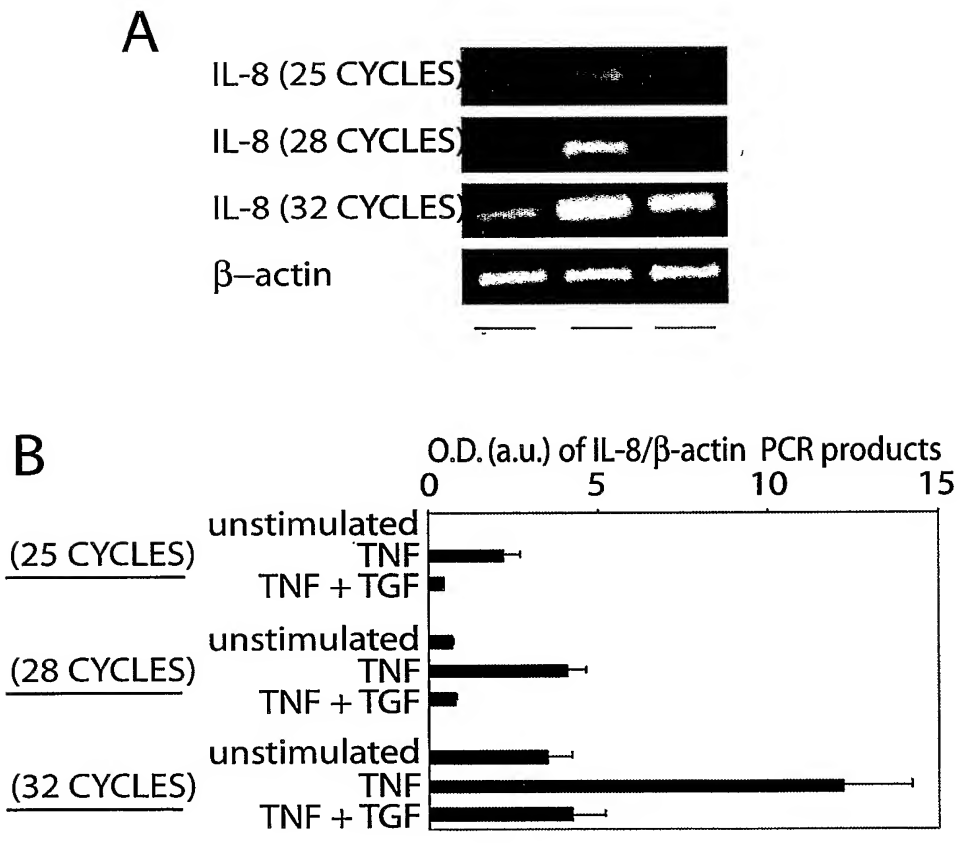

A
IL-8 (25 CYCLES)
IL-8 (28 CYCLES)
IL-8 (32 CYCLES)
β–actin
B
O.D. (a.u.) of IL-8/β-actin PCR products
0
5
10
15
(25 CYCLES)
unstimulated
TNF
TNF + TGF
(28 CYCLES)
unstimulated
TNF
TNF + TGF
(32 CYCLES)
unstimulated
TNF
TNF + TGF

Fig. 10

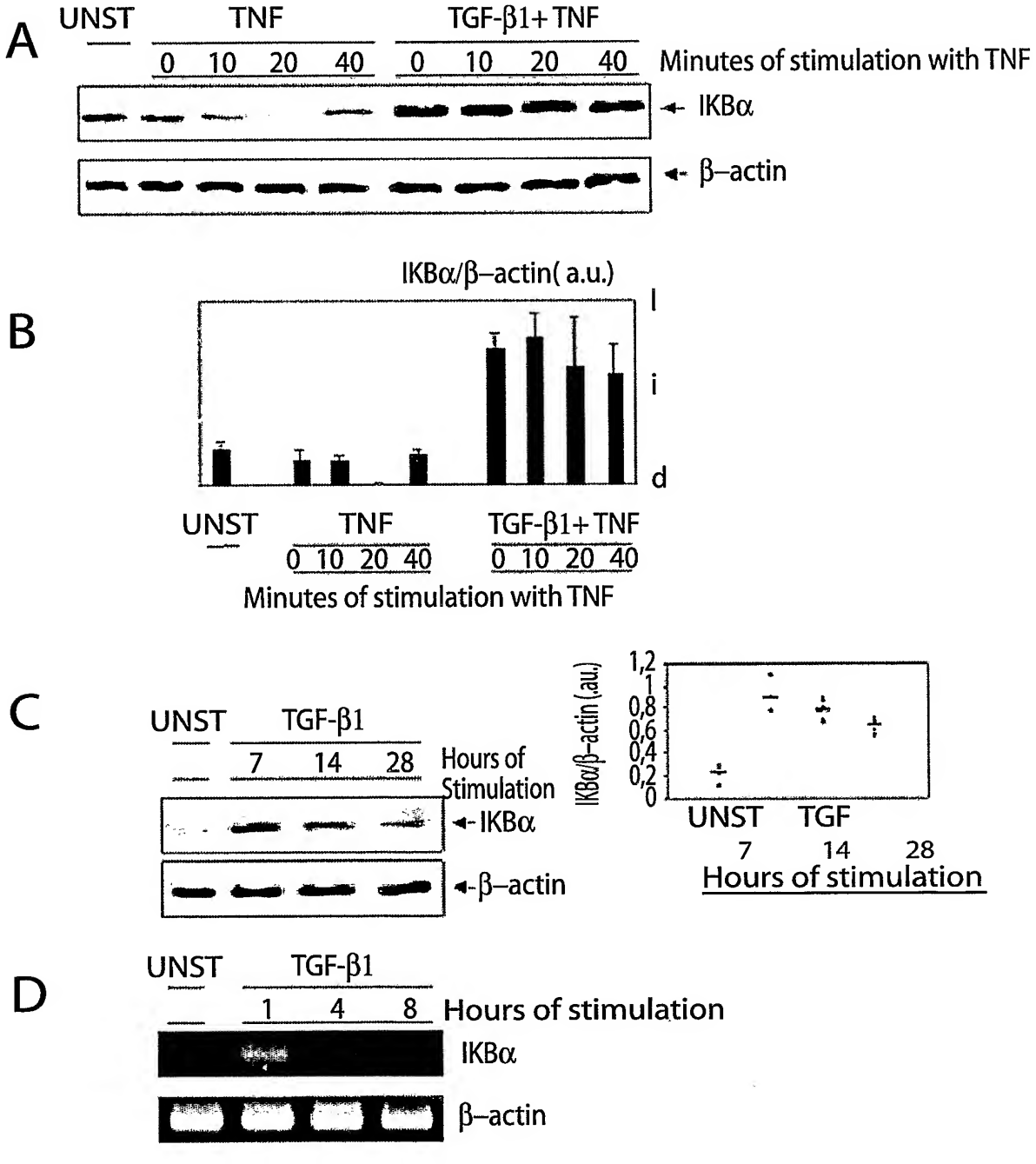

A
UNST
TNF
TGF-β1+TNF
0 10 20 40 0 10 20 40
Minutes of stimulation with TNF
IKBα
β–actin
B
IKBα/β–actin(a.u.)
I
i
d
UNST
TNF
TGF-β1+TNF
0 10 20 40 0 10 20 40
Minutes of stimulation with TNF
C
UNST
TGF-β1
7 14 28
Hours of Stimulation
IKBα
β–actin
IKBα/β–actin (au.)
1,2
1
0,8
0,6
0,4
0,2
0
UNST TGF
7 14 28
Hours of stimulation
D
UNST
TGF-β1
1 4 8
Hours of stimulation
IKBα
β–actin

Fig. 11

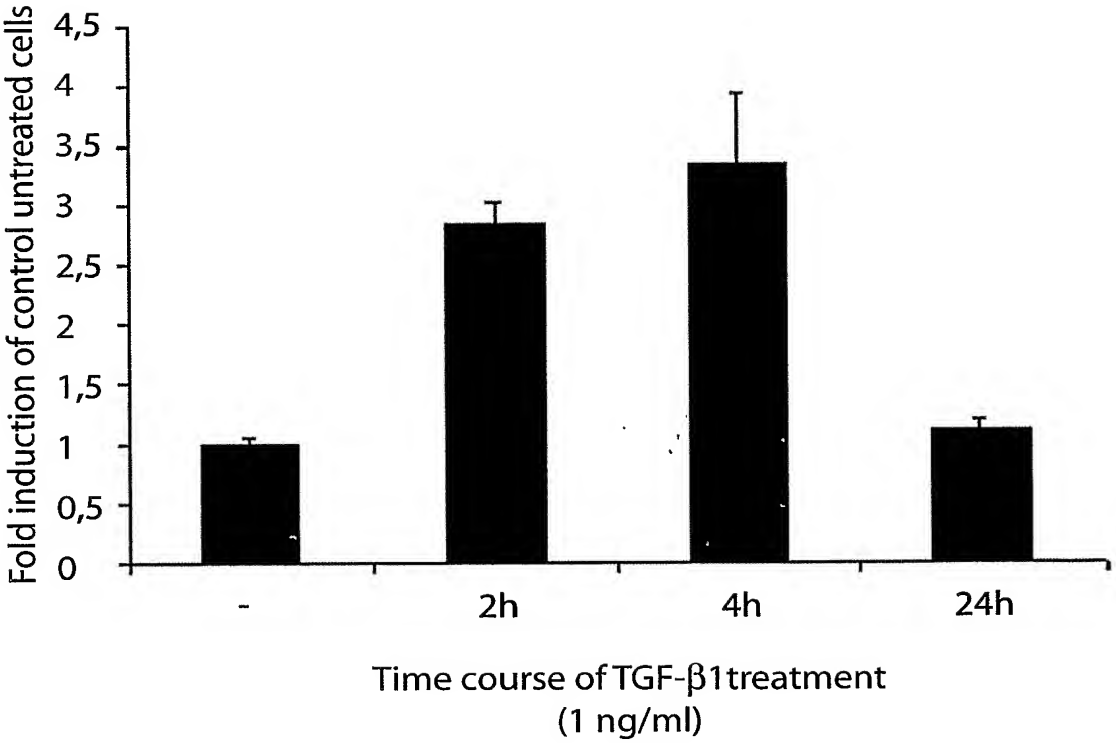

Fold induction of control untreated cells
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
-
2h
4h
24h
Time course of TGF-β1treatment
(1 ng/ml)

Fig. 12

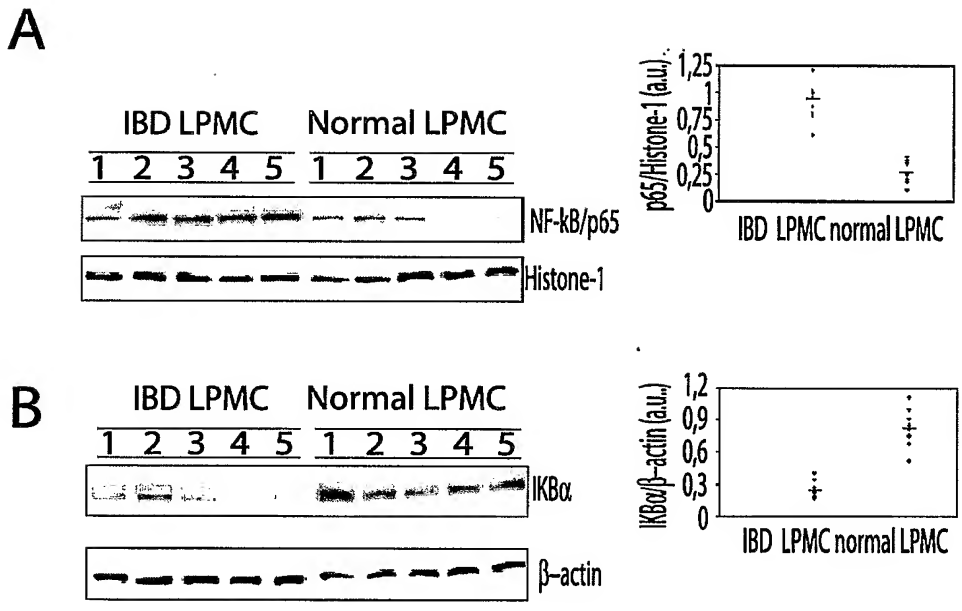

A
IBD LPMC
Normal LPMC
1 2 3 4 5 1 2 3 4 5
NF-kB/p65
Histone-1
p65/Histone-1 (a.u.)
1,25
1
0,75
0,5
0,25
0
IBD LPMC normal LPMC
B
IBD LPMC
Normal LPMC
1 2 3 4 5 1 2 3 4 5
IKBα
β-actin
IKBα/β-actin (a.u.)
1,2
0,9
0,6
0,3
0
IBD LPMC normal LPMC

Fig. 13

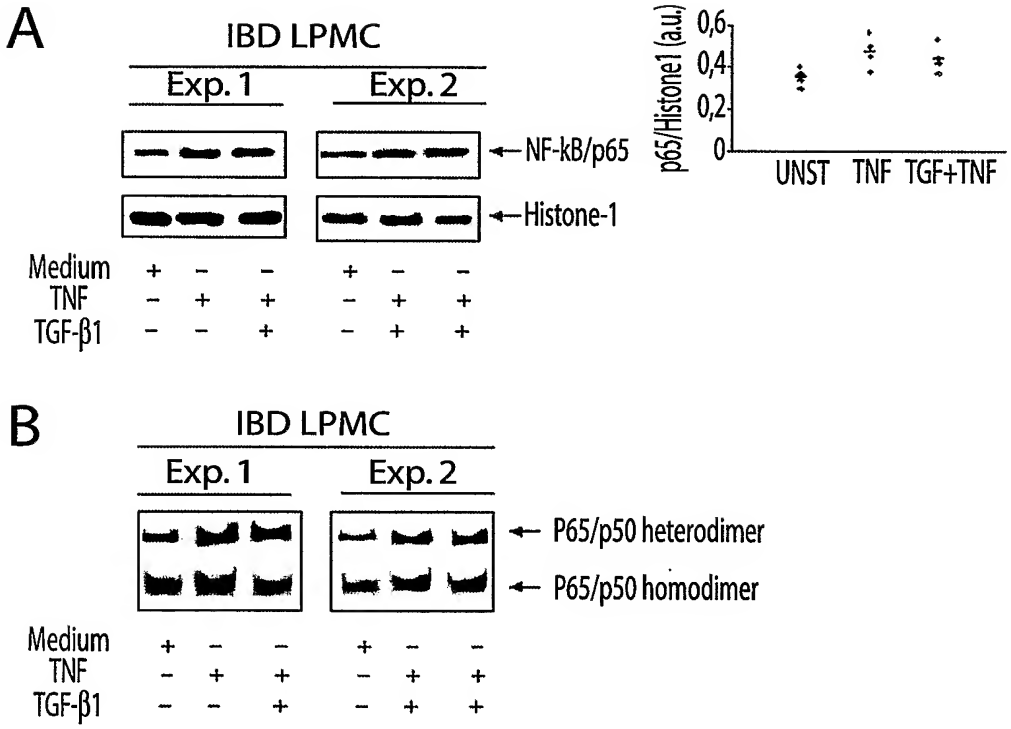

A
IBD LPMC
Exp. 1
Exp. 2
NF-kB/p65
Histone-1
Medium + − − + − −
TNF − + + − + +
TGF-β1 − − + − + +
p65/Histone1 (a.u.)
0,6
0,4
0,2
0
UNST TNF TGF+TNF
B
IBD LPMC
Exp. 1
Exp. 2
P65/p50 heterodimer
P65/p50 homodimer
Medium + − − + − −
TNF − + + − + +
TGF-β1 − − + − + +

Fig. 14

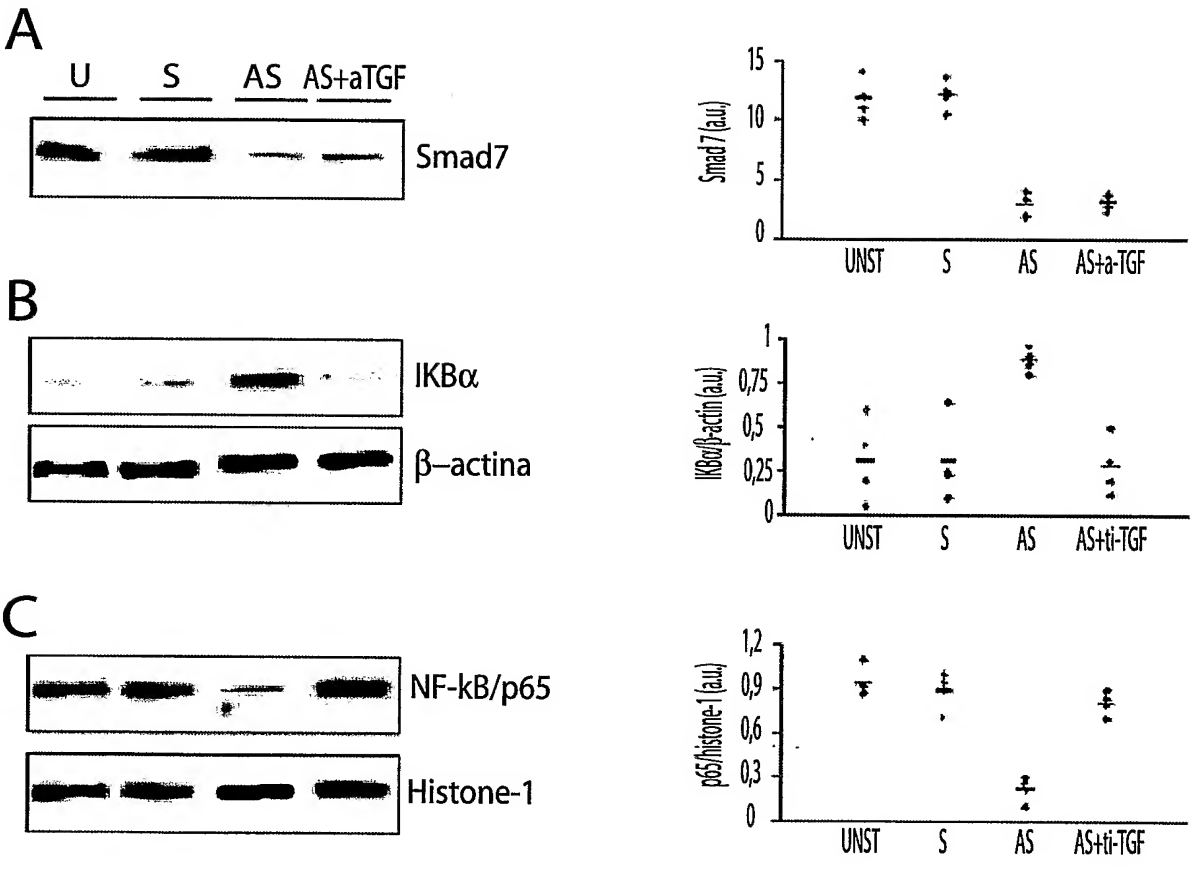

A
U
S
AS
AS+aTGF
Smad7
B
IKBα
β–actina
C
NF-kB/p65
Histone-1
Smad7 (a.u.)
15
10
5
0
UNST
S
AS
AS+a-TGF
IKBα/β-actin (a.u.)
1
0,75
0,5
0,25
0
UNST
S
AS
AS+ti-TGF
p65/histone-1 (a.u.)
1,2
0,9
0,6
0,3
0
UNST
S
AS
AS+ti-TGF

Fig. 15

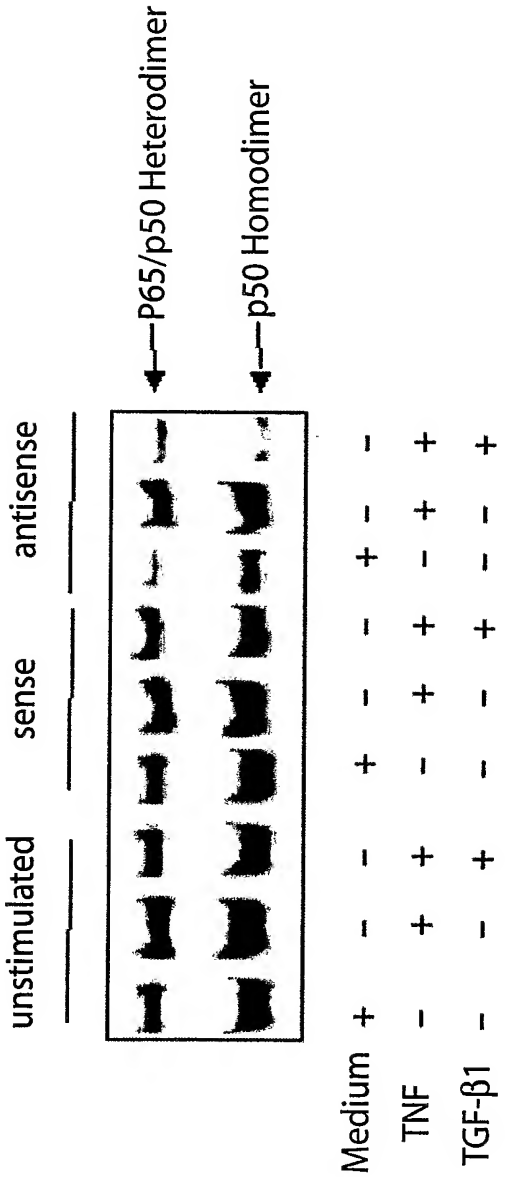

P65/p50 Heterodimer
p50 Homodimer
unstimulated
sense
antisense
Medium + - - + - - + - -
TNF - + + - + + - + +
TGF-β1 - - + - - + - - +

Fig. 16

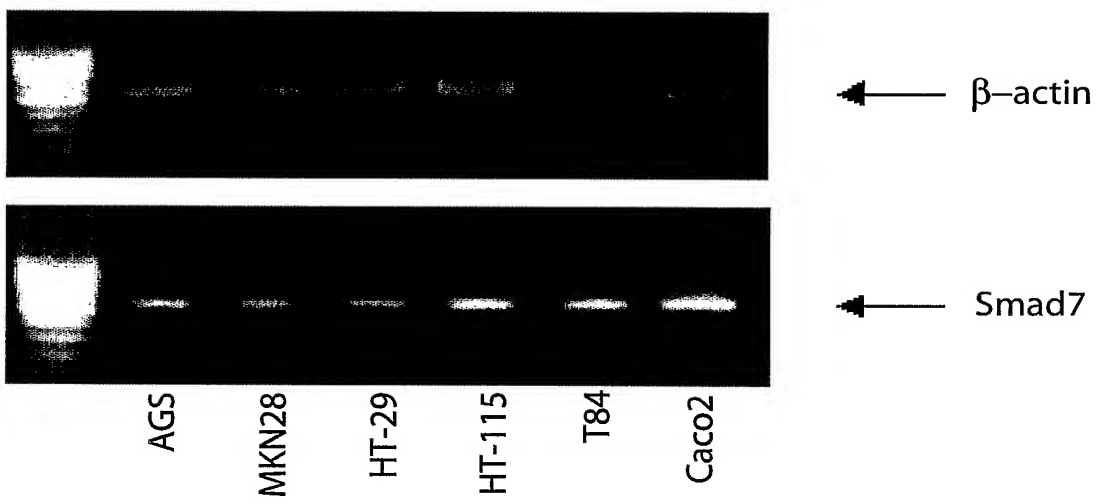


Fig. 17

Giuliani NF-kB.ST25.txt
SEQUENCE LISTING

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<130> PCT 25645

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<141> 2003-08-11

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<151> 2003-08-11

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gagcaca 67

INTERNATIONAL SEARCH REPORT

	ional Application No
	PCT/IT2004/000451

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K31/7115 A61P35/00 A61P1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, PAJ, CHEM ABS Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 159 697 A (COWSERT LEX M ET AL) 12 December 2000 (2000-12-12)	1,11
Y	abstract column 2, lines 8-54 column 5, line 12 - column 6, line 29 column 12, lines 7-29 column 39, lines 39-44 example 15 table 1; sequences ID,NO,15 table 2; sequences ID,NO,15	2-10,13, 14

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 December 2004	10/01/2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Greif, G

INTERNATIONAL SEARCH REPORT

Ir onal Application No
PCT/IT2004/000451

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MONTELEONE G ET AL: "BLOCKING SMAD7 RESTORES TGF-BETA1 SIGNALING IN CHRONIC INFLAMMATORY BOWEL DISEASE" JOURNAL OF CLINICAL INVESTIGATION, NEW YORK, NY, US, vol. 108, no. 4, August 2001 (2001-08), pages 601-609, XP001152527 ISSN: 0021-9738 cited in the application	1,11,12
Y	the whole document	2-10
Y	LANDSTROM M ET AL: "Smad7 mediates apoptosis induced by transforming growth factor beta in prostatic carcinoma cells" CURRENT BIOLOGY, CURRENT SCIENCE,, GB, vol. 10, no. 9, 4 May 2000 (2000-05-04), pages 535-538, XP002252297 ISSN: 0960-9822 the whole document	1,11-13
Y	BITZER MARKUS ET AL: "A mechanism of suppression of TGF-beta/SMAD signaling by NF-kappaB/RelA" GENES AND DEVELOPMENT, vol. 14, no. 2, 15 January 2000 (2000-01-15), pages 187-197, XP002312005 ISSN: 0890-9369 the whole document	1-14
P,Y	MONTELEONE G ET AL: "Smad7 in TGF-beta-mediated negative regulation of gut inflammation" TRENDS IN IMMUNOLOGY, ELSEVIER, CAMBRIDGE, GB, vol. 25, no. 10, October 2004 (2004-10), pages 513-517, XP004581624 ISSN: 1471-4906 abstract; figure 2 page 515, right-hand column, paragraph 3 - page 516, left-hand column, paragraph 1 page 516, right-hand column, paragraph 2	1-14
P,Y	MONTELEONE G ET AL: "INDUCTION AND REGULATION OF SMAD7 IN THE GASTRIC MUCOSA OF PATIENTS WITH HELICOBACTER PYROLI INFECTION" GASTROENTEROLOGY, W.B.SAUNDERS COMPANY, PHILADELPHIA, US, vol. 126, no. 3, March 2004 (2004-03), pages 674-682, XP001183041 ISSN: 0016-5085 the whole document	1,11-14

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ɔnal Application No
PCT/IT2004/000451

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 6159697 A	12-12-2000	AU 2944701 A	31-07-2001
		EP 1248794 A1	16-10-2002
		JP 2003520586 T	08-07-2003
		WO 0153313 A1	26-07-2001
		US 2003049662 A1	13-03-2003